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EXPERIMENTAL CELL RESEARCH

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EXPERIMENTS ON AN ASCITES HEPATOMA

II. INTRAPERITONEAL TRANSPLANTATION OF FREE TUMOR CELLS SEPARATED FROM ISLANDS OF THE RAT ASCITES HEPATOMA

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A morphologically unique type of ascitic tumor, the ascites hepatoma of the rat, has recently been developed by Yoshida and his associates [14]. Originally induced by feeding *p*-dimethylaminoazobenzene (butter yellow) and subsequently transplanted into the peritoneal cavity, the cells of this ascites hepatoma remain in close association following division, forming relatively enormous, free-floating islands. Although other tumors growing in the fluid environment of the peritoneal cavity contain cells which, to a certain extent, exhibit a tendency to form small clusters or "aggregates" [7], such behavior does not compare in magnitude or constancy with the ascites hepatoma. For here, the tumor island, often the product of the association of one hundred or more cells, with characteristics of malignancy equal to those of other ascitic tumors, forms the principal structure of the ascites hepatoma.

Unlike the agglutination reaction characteristic of cells under certain physicochemical conditions, the association of tumor cells in islands of the ascites hepatoma is typically a constant feature, at once more complicated and intimate in its nature. Actually, the tumor island appears to be composed of two morphologically different cells [11]. Thus, while large, round cells constitute the bulk of the tumor island, several crescent shaped cells, resembling endothelial cells, are often seen embedded in the periphery of the island. The peculiar structural characteristics of these cells, combined as it were, in some kind of "symbiotic" [14] relationship, pose a variety of interesting problems as to their individual proliferative capacity and mechanism of division. Further study of such questions, however, depends for its success upon a method by means of which suitable numbers of single tumor cells may be released from confinement within the hepatoma island.

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In the preceding study, the chemical nature of the cementing substance which tends to bind these hepatoma cells together, was investigated with the aid of various enzymes [1]. Apart from crude enzyme mixtures, which appeared to depend for their activity upon the presence of a proteolytic component, only trypsin and chymotrypsin possessed the ability to disaggregate cells of the hepatoma island. From these observations, a relatively simple method was developed, involving the use of trypsin, by means of which tumor islands were converted into suspensions of single cells. In the present study, large numbers of such single tumor cells were transplanted intraperitoneally into new hosts in which the cycle of redevelopment of an ascites hepatoma was followed.

MATERIAL AND METHODS

Tumor material.—Two similar strains of ascites hepatoma (AH 1, No. 7974 and AH 2, No. 130), originally induced by feeding *p*-dimethylaminoazobenzene [4, 11], were used interchangeably in this study and were carried in 3 strains of rats: Marshall 520 (inbred), (Marshall 520 A × C 9935) F_1 hybrid (non-inbred), and a non-inbred stock of Japanese rats [10].

Results of the present study apply equally to either strain of ascites hepatoma, growing in any of the above mentioned strains of rats. There were no significant differences in either the morphology or growth characteristics of either tumor.

As is the case in the Yoshida sarcoma [12], intraperitoneal transplantation of the ascites hepatoma is conveniently performed by direct puncture of the body wall using a thin, sterile, glass pipette drawn from soft glass tubing with an outside diameter of 4 mm. A suitable quantity of tumor ascites is withdrawn in this manner and, from the same pipette, a small sample (about 0.05 cc) is blown directly into the peritoneal cavity of each recipient animal.

Proliferation of both ascites hepatomas is rapid and, within 4 to 5 days following inoculation, the tumor islands typically attain a state of "nearly pure culture" [12]. Although a small percentage of free tumor cells is present, by far the largest proportion is found aggregated in the form of islands, of a wide variety of sizes and configurations. Tumor islands continue to increase in number, with widespread invasion into the peritoneal tissues followed by death of the animal in approximately 2 to 3 weeks [6].

Preparation of free cell suspensions.—Populations of free tumor cells were obtained by incubating tumor islands in the presence of trypsin. Typically, about 0.25 cc of tumor ascites was incubated (1 hour at 37°C) in the presence of 0.1 per cent crude or crystalline trypsin or of 0.1 per cent crystalline chymotrypsin dissolved in 10 ml of 0.1 *M* phosphate buffer at pH 7.3 to 8.0. After one hour of incubation, a mucus-like coagulum, which appeared to contain most of the cellular material, formed in the reaction mixture. Although many free cells were present at this stage, a number of unseparated tumor cell aggregate remained. To completely convert the tumor ascites into a population of free cells, the coagulum (suspended in about 0.25–1.0 cc

of the original solution) which developed after incubation in trypsin was drawn into a syringe, and then forced once or twice through a No. 27 gauge needle. Giemsa stained smears revealed that, by means of this technique, a typical tumor ascites containing islands was readily converted into a suspension in which over 99 per cent of the tumor cells were single.

About 0.25 cc to 1.0 cc of this material, containing single tumor cells, was transplanted into each of several rats. The cells were not counted prior to inoculation. Samples of ascitic fluid, withdrawn from these animals at various time intervals after inoculation, provided a sufficient number of single cells to permit counts.

Inspection of Giemsa stained smears indicated that a number of the inoculated cells had undergone degeneration. That a suitable quantity of viable cells had been initially transplanted, however, was shown by death of the animals and characteristic tumor invasion into the peritoneal tissues in a time and manner similar to that which occurs in the usual transplantation of this tumor.

RESULTS

In a typical experiment a suspension of free tumor cells, in 0.25 to 1.0 cc of phosphate buffer, was transplanted intraperitoneally into each of 5 rats. Giemsa stained smears were made from samples of ascitic fluid withdrawn at 2 hour intervals for a period of 12 hours (Table I and Fig. 1). In a second experiment, samples were similarly obtained at 17, 24 and 48 hours following inoculation (Table II). The data in both tables indicate the average percentages for single tumor cells and tumor cell aggregates counted in the ascites of 5 different rats, together with the approximate standard errors of

TABLE I

Formation of tumor pairs and triples during the first 12 hours after inoculation of single hepatoma cells.[1]

Hours after inoculation of trypsin treated coagulum	No. of animals	Average per cent of single tumor cells	Average per cent of tumor cell pairs	Average per cent of tumor cell triples	Total number of single tumor cells and tumor cell aggregates counted
2	5	99.1±0.2	0.9±0.2	0.08±0.06	13,005
4	5	92.6±1.6	6.3±1.3	1.0 ±0.03	4,996
6	5	74.7±2.7	21.7±2.4	3.6 ±0.8	2,974
8	5	56.6±1.3	35.1±0.6	8.2 ±0.8	2,440
10	5	40.5±1.3	51.5±1.4	8.0 ±1.1	2,925
12	5	36.7±1.7	55.9±1.6	7.4 ±0.8	2,970

[1] Expressed for each cell type as per cent distribution plus estimated standard error of the mean percentage, calculated by Mantel approximation [9].

the mean percentages calculated as range/n [9]. Fig. 1 shows the average per cent changes in the number of tumor cells and tumor cell aggregates during the first 12 hour post-inoculation period, plotted from the data in Table I. The degree of variation at each point is indicated by brackets which represent the highest and lowest percentages obtained from among 5 rats.

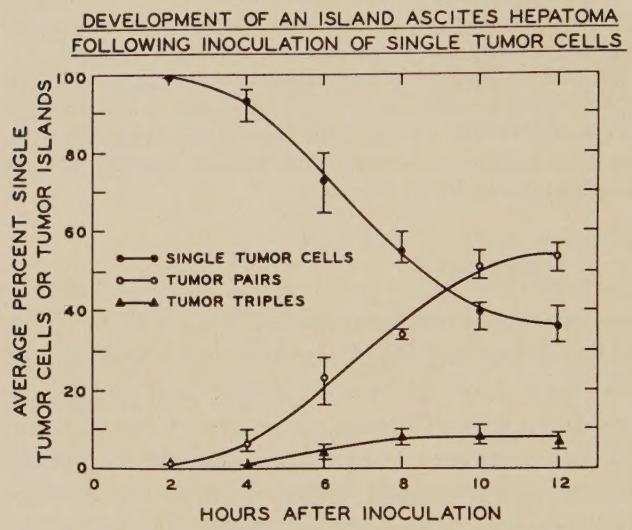

Fig. 1. Development of an island ascites hepatoma following inoculation of single tumor cells

The results clearly demonstrate that the viable portion of the original, single, tumor cell population did not continue to proliferate as free cells, but actually reformed tumor islands. Thus for example, 2 hours after inoculation of the trypsin treated coagulum, free tumor cells represented 99 per cent of the total cell population while the number of tumor cell pairs remained less than 1 per cent (Fig. 1). Although some of these pairs may have failed to separate under the influence of trypsin, it appears probable that most of them were derived from single cells within the period between tryptic separation and the first withdrawal of ascitic fluid. The significant point is that, with time, the percentage of free tumor cells markedly decreased while an almost equivalent rise occurred in the percentage of tumor cell pairs. As indicated in Fig. 1, this process continued until, at 8 to 10 hours after inoculation, the tumor cell population was composed of approximately equal numbers of single tumor cells and tumor cell pairs. At 12 hours post-inoculation, tumor cell pairs had increased to slightly more than 50 per cent of the total population while the percentage of free cells had decreased to somewhat less than 40 per cent. Concomittantly, the percentage

of tumor cell triples (3 cell tumor islands) at 8 hours increased to about 8 per cent and remained at approximately this value for the duration of the experiment.

Similarly, in a second experiment (Table II), examination of the ascitic fluid 48 hours after inoculation of a trypsin treated coagulum showed it to contain about 14 per cent free tumor cells, 42 per cent tumor cell pairs, 17 per cent tumor cell triples and 27 per cent tumor cell islands composed of 4 or more cells. That a certain percentage of cells remain free (14 per cent at 48 hrs.) is not surprising for in this type of ascites hepatoma, originally induced with butter yellow, 10 per cent or less of the population is normally composed of single tumor cells.

TABLE II

Formation of tumor cell aggregates 17 to 48 hours after inoculation of single hepatoma cells.[1]

Hours after inoculation of trypsin treated coagulum	No. of animals	Average per cent single tumor cells	Average per cent tumor pairs	Average per cent tumor triples	Average per cent islands with 4 or more cells	Total no. of single tumor cells and tumor cell aggregates counted
17	5	19.9±2.8	56.3±2.2	14.8±1.4	9.0±0.9	2103
24	5	13.5±1.2	48.4±1.7	21.0±0.8	17.2±2.3	1303
48	5	13.6±0.6	41.5±0.8	17.1±1.2	27.7±2.2	1800

While it may be inferred from these data that reformation of tumor islands from a population of free cells occurs through non-disjunctive cleavage or through cleavage followed by refusion of daughter cells, it is possible that other factors such as reaggregation of free cells also play a role. Thus, for example, preliminary observations have indicated that the formation of tumor islands from single cells may occur even in the presence of colchicine under conditions in which normal mitosis has been almost completely blocked. On the other hand we have also observed formation of tumor pairs and triples under *in vitro* conditions in which normal mitosis proceeded but reaggregation of free cells was reduced to a minimum. This was accomplished by first mixing the cell suspension with 50 per cent chick embryo extract, adding to this a small quantity of chick plasma and allowing the resultant mixture to clot on a cover slip. The preparation was then bathed in ascitic fluid at 37°C for a period of two days.[2] During this interval rest-

[1] Expressed for each cell type as per cent distribution plus estimated standard error of the mean percentage. Calculated by Mantel approximation [9].

[2] We are indebted to Dr. Joseph Leighton and Mr. Ira Kline for the culture preparations used in these experiments.

ing tumor cells embedded in the plasma clot remained free while those cells entering mitosis proceeded to form pairs and triples. Apparently then, both normal mitosis and reaggregation of single cells participate in the reformation of an island ascites hepatoma.

Our results demonstrate only that a free cell population is not produced under the conditions of these experiments.

The relative ease with which the initial development of the ascites hepatoma could be followed, encouraged us to investigate further the nature of the crescent shaped cell which often seemed to form so intimate a part of the tumor island. While Yoshida and his associates have described the presence of such cells ("endothelial") in tumor pairs and large islands, the material they used was a well developed ascites hepatoma several days after transplantation [11, 14]. In the present experiments, however, the development of tumor pairs was followed for several hours immediately after their formation from newly divided single cells. Under these conditions the majority of tumor pairs which developed within a period of several hours following inoculation of single cells, possessed no crescent cell. Indeed, both cells were generally of equal size, round in shape, appearing in most respects like hepatic cells. With time, however, an increasing number of crescent shaped cells were observed and within 8 to 12 hours post-inoculation, a large number of tumor pairs contained such cells.

To resolve these apparent variations in structural appearance and the presence or absence of a crescent cell, one might assume that the "young" hepatoma pair (Figs. 6–8) undergoes a series of morphological alterations following its formation from a newly divided cell. Such changes would lead finally to formation of a "mature" pair containing both hepatic and crescent shaped cells (Figs. 9–12). Observations in the present study suggest that such a phenomenon actually occurs. Indeed, a large majority of the several thousand tumor cell pairs counted on Giemsa stained smears could be placed in each of several categories depending upon the degree of transformation to which they had progressed (Figs. 6–12). It should be understood, however,

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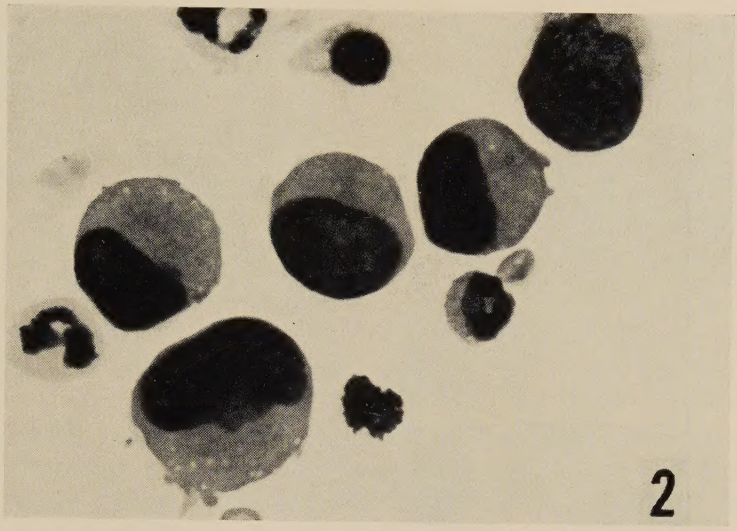
Figs. 2–14. Giemsa stained smears of the ascites hepatoma after inoculation of single cells. (Figs. 2–5, a few hours post-inoculation; Figs. 6–14, 12 hours post-inoculation). Mag. about 1000 ×.
Fig. 2. Isolated hepatoma cells a few hours after inoculation.
Figs. 3–5. Prophase, metaphase and telophase in single hepatoma cells a few hours after inoculation.
Fig. 6. Tumor pair in early stage of association.
Fig. 7. Tumor pair in early stage of association with beginning of vacuolization.
Fig. 8. Tumor pair in early stage of association. Note vacuoles on both sides of mid-line.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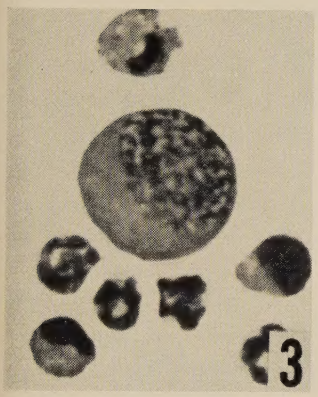
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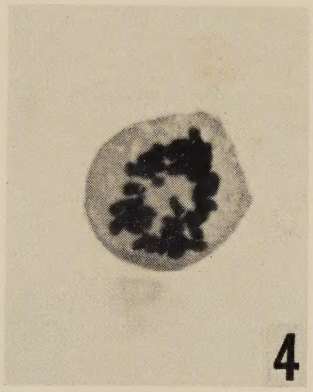
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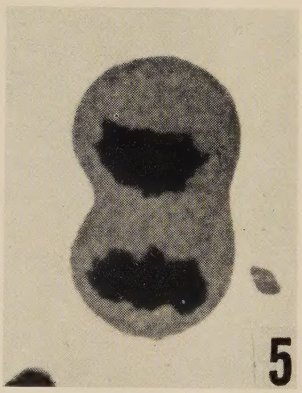
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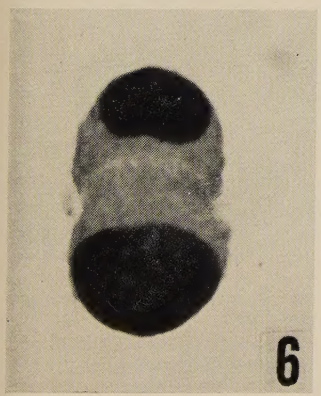
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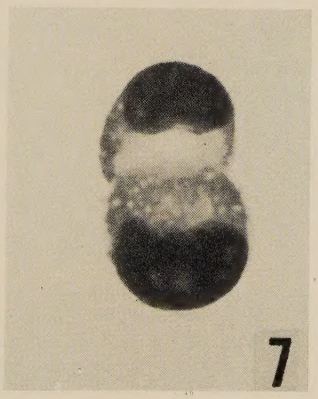
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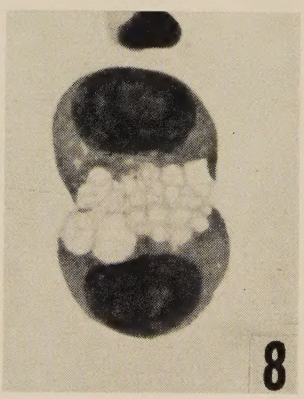
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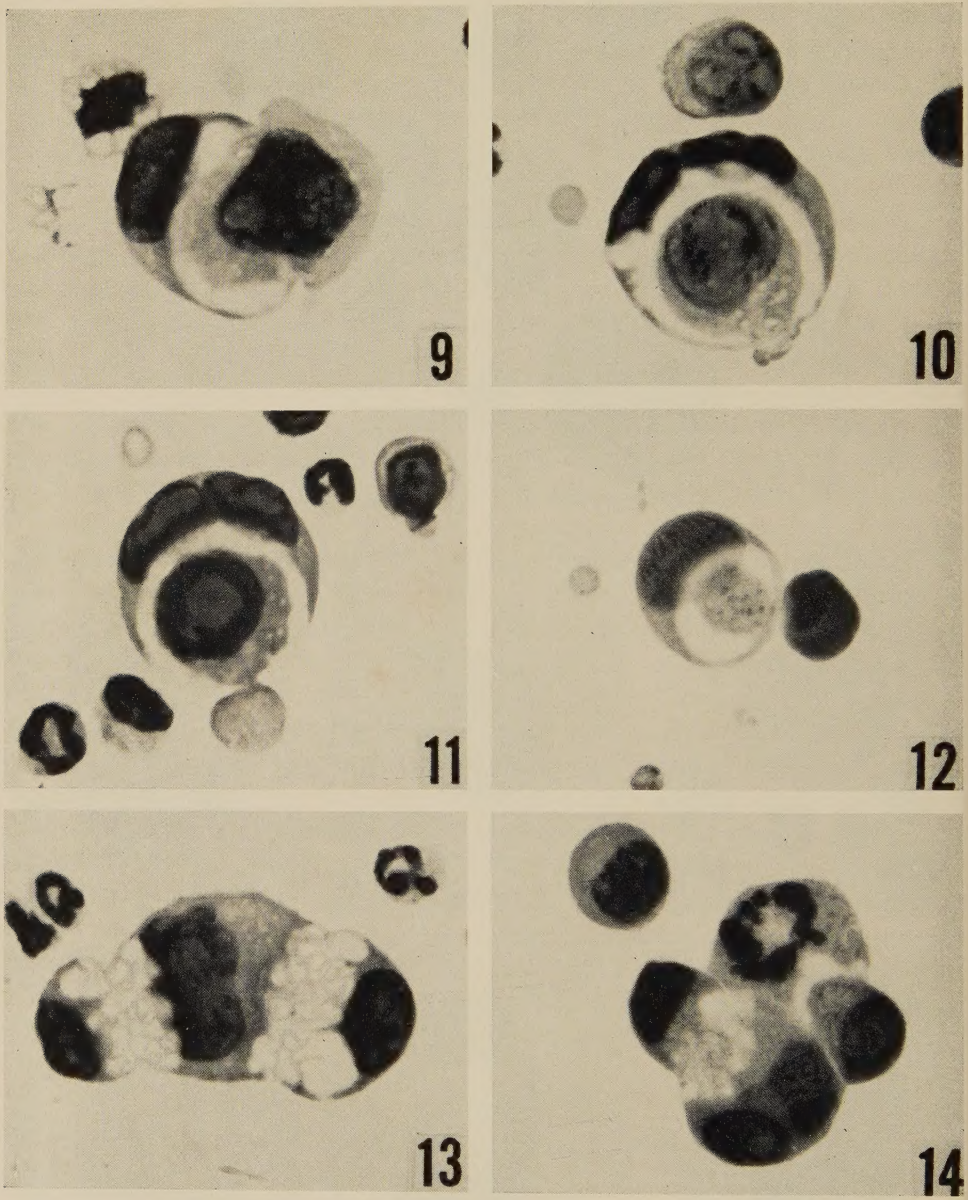

Fig. 9. Intermediate stage in transformation of tumor pair. Cell on left has assumed a crescent (endothelial-like) form and is proceeding to pinch off or engulf the other cell.
Fig. 10. Final stage in transformation of tumor pair. One cell has completely engulfed the other.
Fig. 11. Final stage in transformation of tumor pair. One cell has succeeded in dividing the other into two segments. A portion of protoplasm has been pinched off.

that this concept is essentially a working hypothesis, and not yet completely established.

Apparently, an initial step in the formation of a hepatoma pair is the failure of the daughter cells to separate completely following their formation from a newly divided cell (Fig. 6). Secondly, a disturbance of some kind, often marked by the appearance of numerous vacuoles in the surrounding protoplasm, occurs at the original line of cleavage (Figs. 7–8). Following this, one cell of the hepatoma pair proceeds to elongate and, guided by the surface curvature of its partner, finally assumes a crescent-like shape (Fig. 9). From the tips of this crescent, "protoplasmic arms" extend around the partner cell in a kind of "encircling" process which may lead to complete engulfment (Fig. 10). Very often, however, this phenomenon is somewhat modified and leads instead to a kind of "pinching off" process which results in a partial or complete severance of the partner cell. Three examples are illustrated in Figs. 9, 11 and 12. Thus, in Fig. 9 this "pinching off" process is half complete and this type of pair is often seen during the normal course of transplantation. In Fig. 11 one cell has been divided unequally into a nucleated segment which is contained within the crescent shaped cell, while a portion of protoplasm, attached by a short strand, remains outside. A similar case, but one in which the nucleus has been "clipped off" is illustrated in Fig. 12.

While this phenomenon appears to occur in a large number of cases, it is not necessarily essential to the formation of all tumor islands in the ascites. Thus for example, Figs. 13 and 14 illustrate a tumor triple and a 4-cell tumor island which do not possess crescent shaped cells. Again it should be pointed out that this process described in Figs. 6–12 appears to occur not only in tumor pairs formed through mitosis but also in pairs which were united through reaggregation of single cells.

DISCUSSION

Of the interesting cellular processes responsible for the formation of the hepatoma island, the behavior of the crescent shaped cell has primarily

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Fig. 12. Final stage in transformation of tumor pair. Similar to Fig. 11 but in this case the nucleus has been pinched off.
Fig. 13. A tumor triple.
Fig. 14. A 4-cell tumor island. Upper cell is in mitosis.

engaged the attention of earlier investigators. Actually, this cell is synonomous with the so-called endothelial cell suggested by Yoshida and his associates as bearing some relation to the origin of the Yoshida ascites sarcoma [11, 14].

Briefly, the line of reasoning established through previous investigations is as follows: the Yoshida ascites sarcoma arose unexpectedly in one of 20 rats fed with *o*-aminoazotoluene and then painted on the dorsal surface of the skin with a solution of potassium arsenite; a regimen usually employed for the production of hepatoma [13, 14]. While the result was the appearance of a cirrhotic liver bearing small, multiple hepatoma nodules, the peritoneal cavity contained a thick, milky white ascites; a consequence of the presence of numerous tumor cells. It is from these cells that the present strain of Yoshida ascites sarcoma was derived. Attempts to duplicate these results led eventually to the development of another strain of "Yoshida sarcoma" (MTK sarcoma), indistinguishable from the original, in a rat fed first for 300 days on *o*-aminoazotoluene and then on *p*-dimethylaminoazobenzene, without the application of potassium arsenite [8]. Thus, the origin of the Yoshida ascites sarcoma was assumed to be associated with the development of aminoazo dye hepatoma.

Based upon histological studies of rat hepatoma, induced by feeding *o*-aminoazotoluene, Yoshida and his associates suggested that the endothelial cell often appeared capable of neoplastic transformation and proliferation and might be a possible source from which the cells of the Yoshida ascites sarcoma originated [14].

Upon conversion of the solid hepatoma into an ascites form, the "endothelial" cell was identified as a crescent shaped cell which appeared to be in intimate association with hepatic cells in a majority of tumor pairs and at the surface of many of the larger islands [11, 14]. While an earlier study [2] indicated that two different kinds of cells, hepatic and "endothelial" (crescent shaped), were contained within an island of the ascites hepatoma, a recent reinvestigation of the question by Isaka *et al.* has led to an altered viewpoint [3]. For they have shown that a single hepatic cell, inoculated intraperitoneally, reforms a new, typical, island ascites hepatoma which contains both types of cells.

These observations can readily be explained and confirmed on the basis of the results reported in our study. Thus, we have been able to demonstrate, by means of a somewhat different approach, that no cell of this ascites hepatoma is capable of continued proliferation as a free cell tumor. Instead, the free cell population is, at first, progressively converted into

tumor pairs which later proceed to develop into successively larger aggregates until finally, a typical island ascites is reformed. Accompanying this process and particularly evident in tumor pairs, is perhaps a more important phenomenon in which one cell engulfs or severs its partner in half and at the same time becomes structurally transformed into a crescent shaped cell. The final result is the presence, in hepatoma islands, of two morphologically different cells which nevertheless possess a common origin. In further support of this view are the recent, *in vitro* observations of Kaziwara *et al.* [5], who followed the direct transformation of a hepatic cell into a crescent shaped ("endothelial") cell, through a series of structural changes essentially similar to those reported in the present study.

SUMMARY

1. Large numbers of single tumor cells were obtained by a relatively simple procedure based on the ability of trypsin to digest the intercellular cement substance in the islands of the ascites hepatoma. Populations of single cells were then transplanted intraperitoneally into new hosts, where the cycle of redevelopment of a new ascites hepatoma was followed.

2. The viable portion of the original, single tumor cell population did not continue to proliferate as free cells, but reformed tumor islands. Thus, 2 hours after inoculation of the trypsin treated coagulum, free tumor cells represented 99 per cent of the total cell population while the number of tumor cell pairs remained less than 1 per cent. Within 8 to 10 hours after inoculation the tumor cell population was composed of approximately equal numbers of single tumor cells and tumor cell pairs. In a second typical experiment, examination of ascitic fluid was made at intervals from 17–48 hours after inoculation. The count at 48 hours showed about 14 per cent free tumor cells, 42 per cent tumor cell pairs, 17 per cent tumor cell triples and 27 per cent tumor cell islands of 4 or more cells.

3. Observations of several thousand tumor cell pairs counted on Giemsa stained smears indicated that a hepatoma pair may frequently undergo a series of morphological alterations following its formation from a newly divided single cell. The end result is the formation, in many cases, of a tumor pair possessing one crescent-like cell ("endothelial") and one hepatic cell.

4. The relation of the crescent-like cell to the "endothelial" cell suggested by Yoshida and his co-workers as being implicated in the origin of the Yoshida ascites sarcoma is discussed.

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MICROELECTRODE EXPERIMENTS ON UNFERTILIZED SEA URCHIN EGGS[1]

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Information on the electrophysiological properties of eggs has been obtained by measurements on eggs in suspension [1, 2, 3, 4, 6]. Difficulties have, however, been encountered in evaluating the membrane resistance of the egg; moreover, in experiments of this kind, the membrane potential cannot be measured. This could best be done by recording directly over the membrane after insertion of a microelectrode into the egg. This experiment was tried by Rothschild [5], using microelectrodes having a tip diameter of down to 2 μ. He found no resting potential nor any change in potential during fertilization. Recently, it has again been reported [7] that there is no potential over the membrane of the unfertilized egg.

The present investigation is confined to the electrical properties of the unfertilized egg. It will be shown that an attempt to introduce a microelectrode into an egg meets with difficulties. The membrane resistance has, however, been measured.

METHODS

The surface and deep-water form of eggs from *Psammechinus miliaris* were used (Kristineberg, Swedish west coast). The experiment was performed with the eggs set in an agar gel (1.5 per cent agar in sea water). The agar solution was cooled with stirring and the eggs were introduced during stirring at 28°C. A few seconds later, the agar solution was allowed to solidify at 4°C.

Conventional glass capillary electrodes filled with 3*M* KCl solution and with a tip diameter of less than 0.5 μ were used. The potential over the tip of the microelectrode, measured on samples of electrodes, was less than 3 mV, and the resistance varied between 2 and 30 mΩ. For recording, a dc-amplifier and a cathode ray tube were used. The input stage was a cathode follower (RCA 954). The grid current was adjusted to less than 5×10^{-11} A. The microelectrode was connected to the input stage through an Ag–AgCl electrode; in the agar with the eggs, a large Ag–AgCl electrode was connected to earth via a small resistance, through which a calibration voltage could be applied.

In most experiments, a second microelectrode could be inserted, and a switch allowed recording from or polarization through either electrode. For polarization, a voltage was applied between the microelectrode and an Ag–AgCl electrode in the

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agar. The strength of current was determined by measuring the drop of potential over a calibrated resistance in series with the electrodes. After amplification, the voltage was read on a moving-coil galvanometer. To allow measurement, the minimal time of current flow was one second.

Some experiments were made with eggs without vitelline membrane; it had been removed by treatment with sea water containing 0.00025–0.0005 per cent chymotrypsin.

RESULTS

When a microelectrode was inserted into the egg, the microelectrode became positive in relation to the other electrode. In the great majority of experiments, this positivity did not exceed 5–10 mV, but in eggs from some animals potentials up to 40 mV were recorded. The potential shift was not sudden (Fig. 1), as when a microelectrode is inserted into a muscle fibre. Moreover, it was observed in most cases that the positive potential could be established by degrees by insertion step by step. However, with further insertion, the positivity did not increase, but suddenly fell to zero when the microelectrode had passed through the egg. It was sometimes found that the electrode tip could pass more than 100 μ beyond the egg before the potential suddenly jumped to zero; the surface layer of the egg was apparently drawn out into a pouch. If the tip of the electrode was only slightly damaged, the electrode could be inserted into the egg without any shift in the potential.

It cannot be assumed that the positive potential established on insertion of an undamaged electrode is recorded over the membrane of the egg. In order to study this question, two electrodes were thrust into an egg. When the electrode tips were undamaged, the same potential was usually recorded. If the tip of the electrodes had penetrated the membrane, it could be expected that, with current through one electrode, the other electrode would record a shift in the membrane potential. However, in the great majority of experiments, it was found that when current was passed through one electrode, there was no or very little change in the potential of the other electrode. This indicated that the latter electrode had not penetrated the cell membrane.

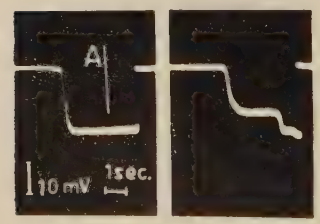

Fig. 1. Potentials established on passing a microelectrode through an egg. To the right is seen the result when the electrode enters the egg step by step. The downward deflection of the beam microelectrode is positive. *A* shows the axis of beam deviation.

An attempt was made to continue the analysis in the following way. The tip of the microelectrode inserted deeply into the egg has to be in contact only with a very minute area of membrane. Passage of fairly strong current through the microelectrode could be expected to destroy this part of the membrane, thereby permitting intracellular recording. When current ($0.46–1.3 \times 10^{-7}$ A) had been passed through both electrodes inserted into the cell, it was, in fact, found in a number of cases that when a weak current was applied through one electrode, a potential shift could be measured with the other electrode. The potential of the cell interior could be varied in this way between +300 and −300 mV. In order to determine the membrane resistance, a weak current that changed the membrane potential by 10–40 mV was applied, and measurements of current and membrane potential shift allowed the calculation of membrane resistance. In eggs from the surface form of *Psammechinus miliaris*, the transverse resistance of the membrane ranged from 900 to 1650 ohm. cm², with a mean value (15 determinations) of 1350. In eggs from the deep-water form, the corresponding values were 1100 to 2400 ohm. cm², with a mean value (15 determinations) of 1700.

It could be inferred from the preceding that, after a comparatively strong current had been passed through the electrode, the potential of the cell interior could be recorded. It therefore appeared likely that the potential measured would give the value of the normal membrane potential. This did, however, not seem to be the case. In some instances this electrode was still positive to the outside electrode, but in the majority of cases the electrode through which current had been passed recorded a reversed potential, i.e., it became

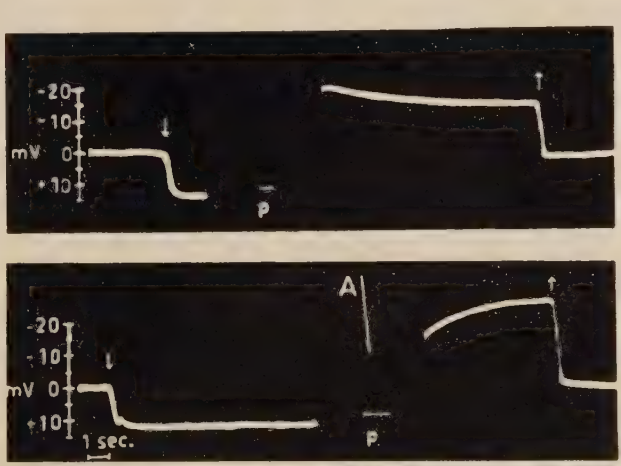

Fig. 2. The effect of current through a microelectrode inserted into an egg on the potential recorded by this electrode. Microelectrode inserted at downward arrow, withdrawn at upward arrow. Horizontal line (*p*) denotes duration of current flow; in the upper record 9.0×10^{-8} A, in the lower record 7.2×10^{-8} A. *A* shows the axis of beam deviation.

negative instead of, as earlier, positive, as illustrated in Fig. 2. The negativity sometimes amounted to 30 mV and decreased with time. It was often found to reach a maximum some time after the end of polarization. Its incidence was the same after current in either direction through the microelectrode. It is noteworthy that a second electrode, inserted into the egg after an equally strong current flow through it, could record a very different potential, even if the tip, judging by the resistance measurements, also recorded from the cell interior. A possible explanation of this finding is that the current flow through the tip of the electrode caused local cytolysis, with an intracellular potential gradient bound to the semisolid structure of the cortex.

The question then arises whether any conclusions regarding the value of the membrane potential and resistance can be drawn from the experiments just described. With regard to the former, what is the relation of the potential established on insertion of the electrode to the potential over the membrane? With regard to the latter, has the relatively strong current passed through the egg to permit the measurement changed the properties of the cell membrane? These questions can be answered on the basis of a few experiments in which the tip of the microelectrodes appeared, in fact, to penetrate the membrane on insertion. Thus, in one experiment (surface form), 5 mV positivity was recorded with either of two electrodes inserted into the egg. 4.55×10^{-9} A was then applied through one electrode and the potential of the other was decreased by 22 mV. Current through the latter electrode shifted the potential of the first by the same amount per unit current. Since the diameter of the egg was 120 μ, the transverse resistance of the membrane was 2200 ohm. cm^2, and this value is in good agreement with the aforementioned finding. Thereafter, 1.0×10^{-7} A was allowed to flow for one second through one of the electrodes, and the resistance measurement was repeated with a weak current. The resistance was then found to be 1900 ohm. cm^2.

Consequently, no appreciable change in the membrane resistance seemed to have resulted from the relatively strong current flow through the microelectrodes that was, as a rule, necessary to permit the measurement. Since the experimental procedure tends to decrease the resistance, the figures may be somewhat lower than in the intact egg.

Also in these experiments in which resistance measurements could be made without preceding strong current flow, the microelectrodes became about 5 mV positive on insertion. This finding may indicate that the membrane potential is of this magnitude with the interior of the egg position, but the observations are too few to allow any conclusion.

Experiments were also made on eggs after removal of the vitelline membrane with chymotrypsin. The results did not differ significantly from those described above.

COMMENTS

The analysis of the electrophysiology of the sea urchin egg has proved difficult. It is noteworthy that the microelectrode does not penetrate the plasma membrane of the egg, although a potential is recorded when the electrode is inserted. Unless the potential is established by a jump, it is not permissible, without further experiments, to conclude that the tip has penetrated a membrane. The failure of the electrode to penetrate the membrane is not necessarily to be ascribed to any properties of the plasma membrane itself; it may very well be due to the presence of the underlying semisolid cortex. In the present investigation, no attempt has been made to study possible changes in membrane resistance and potential on fertilization. This problem necessitates recording of the intracellular potential; as described above, this may be achieved after a short pulse of current through the recording electrode. However, if the current flow also brings about a potential gradient within the cytoplasm in the immediate vicinity of the tip, and changes in the solidity of the cortex take place on fertilization, a factor of uncertainty would be introduced with the use of this method.

SUMMARY

A microelectrode inserted into an egg of *Psammechinus miliaris* is found to exhibit 5–10 mV positivity to an indifferent electrode. With two microelectrodes inserted into an egg, a current flow through one of them does not usually shift the potential of the other. It is concluded that the tips of the electrodes have not penetrated the membrane.

The membrane resistance was measured after passing a comparatively strong current through two electrodes inserted into the egg, probably resulting in local destruction of the part of the membrane in contact with the tip of the electrodes. The mean value of membrane resistance was 1350 ohm. cm^2 in eggs from the surface form, and 1700 in eggs from the deep-water form. It is concluded that plasma membrane resistance of the unfertilized egg is of this order of magnitude or possibly somewhat higher.

This investigation was aided by a grant from the Swedish Medical Research Council. Technical assistance was given by Miss R. Araldsson, which is gratefully acknowledged.

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ON THE PROPAGATION OF CORTICAL RESPONSE OF THE SEA URCHIN EGG TO ELECTRICAL STIMULATION

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Very little is known on the effect of electrical stimulation of animal cells. Stimulation of ameba by electric shock causes a liquefaction of the cortex [1], and Yamamoto observed the breakdown of the cortical alveoli and subsequent membrane elevation on ripe unfertilized egg of the fish, *Oryzias latipes* [14]. It is clear that the concentration of certain ions at the protoplasmic membrane facing a certain electrode is responsible for the partial cytolysis. In view of Loeb's theory [3], that the reaction of activation of the egg consists of a transient cytolysis, it seemed interesting to check whether a cytolytic reaction caused by an electrical shock would be able to bring about activation phenomena.

As a result of the electrical stimulation of the sea urchin egg with direct current, Moser [6] described partial membrane elevation. If, however, the egg were submitted to a preliminary treatment with oxalate or citrate no membrane formed and cytolysis resulted. The electrical stimulation used in his experiment seems to be too strong: in fact no clear membrane elevated and complete cytolysis rapidly ensued. Theoretically, one would expect that if an adequate current is applied, the partial cytolysis initiated at one point should spread over the whole surface of the egg and membrane elevation should take place.

Sugiyama [9, 10, 11] has emphasized the important role of Ca in the fertilization reaction and [12] also observed that the propagation of the response to stimulating reagant was inhibited by urethane. It might be expected therefore that the response to an electrical shock would be different in the absence of Ca or after a preliminary treatment with urethane.

In the present paper some results will be reported which have been obtained on the effect of an electrical stimulation with direct current on the membrane elevation in sea urchin eggs in normal and Ca- and Mg-free artificial sea water and after treatment with sodium oxalate and urethane.

[1] Present address.

MATERIAL AND METHOD

The experiments were carried out on mature unfertilized eggs of *Hemicentrotus* (*Strongylocentrotus*) *pulcherrimus* at Sugashima Marine Biological Station of Nagoya University, Mie-ken, Japan during the winter 1953.

Non-polarisable Zn-$ZnSO_4$ electrodes were used the bottom of which was made of a piece of earthware. A suspension of about thirty eggs was placed in the centre part of a capillary tubing having a length of 3 cm and an inner diameter of 0.4–0.3 mm. After sealing of both ends of the capillary with 2 per cent agar made up in sea water without air-bubbles, contact of the sealed ends of the capillary with the electrodes was established through a bridge of cotton soaked in sea water.

The direct current was supplied from a constant voltage apparatus equipped with vacuum tubes. The circuit was also equipped with a rheostat (5 kiloohms) and a milliampermeter. The electric resistance of the circuit was from 80 to 100 kiloohms and was measured with a Whitestone bridge after the stimulation of the egg. The potential gradient of the electrical field was calculated from the formula:

$$\text{P. G.} = iR/S$$

where i is the current of the circuit; S the area of the cross section of the capillary; R the specific resistance of the medium which was also calculated from its conductivity. The potential gradient applied in the present experiments was from 5.6 to 8.9 volts per centimeter.

As the apparatus for closing and, after a short while, breaking the circuit, the modification of *Lucas's pendulum* was applied. Each shock lasted from 1/20 to 1/100 second, and was measured from the photographs taken on the screen of a X-ray oscilloscope.

OBSERVATIONS

Electrical stimulation in sea water.—For weak electrical shock (5.6 volts/cm, $^1/_{20}$ sec.) partial membrane elevation took place on the side of the egg facing the anode. The thickness of the membrane did not seem to be different from that of a normally fertilized egg and the perivitelline space was filled by blisters which protruded out of the egg (Fig. 1). When a stronger electrical field was applied (6.7 volts/cm, $^1/_{20}$ sec.), the membrane elevated, first, at the anodal and then at the cathodal side (Fig. 2). On the anodal side, the previtelline space was not only wider than that of the cathodal side but also wider than that appearing at the anodal side of an egg stimulated to a weak shock. Such results show that an electrical shock is able to cause membrane formation and cytolysis on both the anodal and cathodal sides of the egg. Moreover the anodal effect appears first and is stronger than at the cathodal side. At the side of membrane elevation the cortical granules had broken down whereas on the rest of the egg surface they still

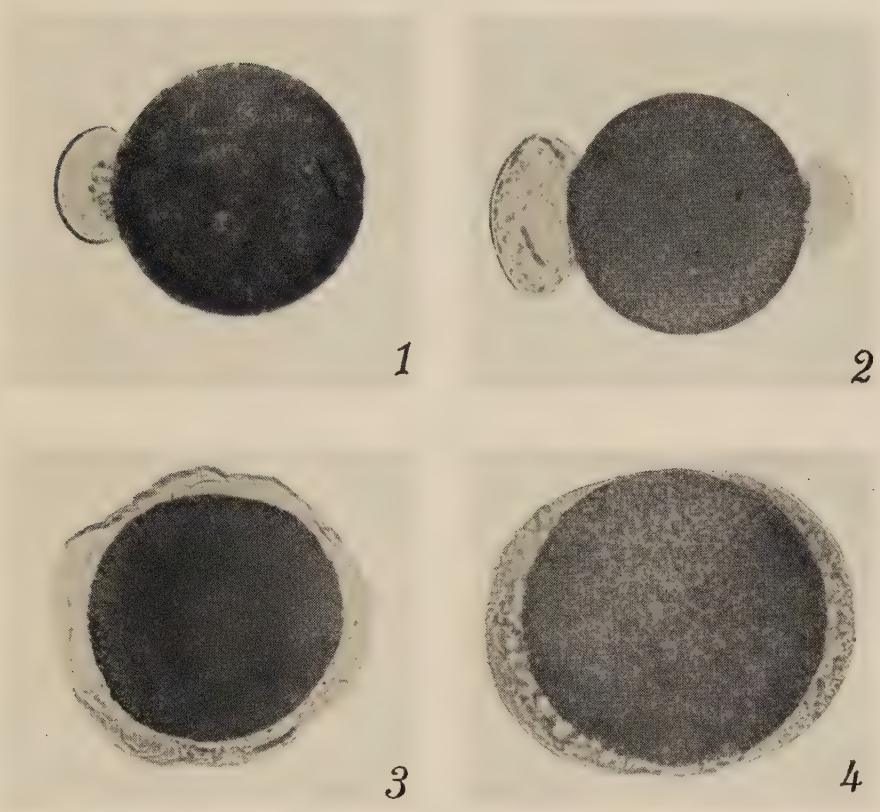


Fig. 1. Egg stimulated with a weak electrical current in sea water.
Fig. 2. Egg stimulated with a stronger current than in the case of Fig. 1.
Fig. 3. Membrane formation when the egg of Fig. 2 was inseminated.
Fig. 4. Egg stimulated with a still stronger current. Anodal side; left.

existed. If such eggs were inseminated, normal membrane formed on the rest of the egg surface (Fig. 3). The egg, however, became polyspermic.

Eggs placed in a still stronger electrical field (7.2 volts/cm, $^1/_{20}$ sec.) formed an elliptical membrane (Fig. 4). In some cases, the membrane did not completely elevate on that part of the egg which was at a right angle to the direction of the electrical current. Very few eggs with normal concentric circular type of membrane were observed. In all these eggs the perivitelline space was filled by protoplasmic blisters and no hyaline layer formed. Moreover, no signs of activation were observed.

If the potential gradient was too strong then the eggs underwent cyto-

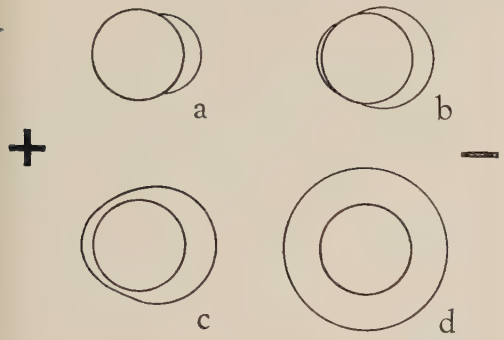
lysis. In the course of cytolysis, the eggs flattened at the cathodal side, ruptured and the internal granules escaped, just as described by Moser [6].

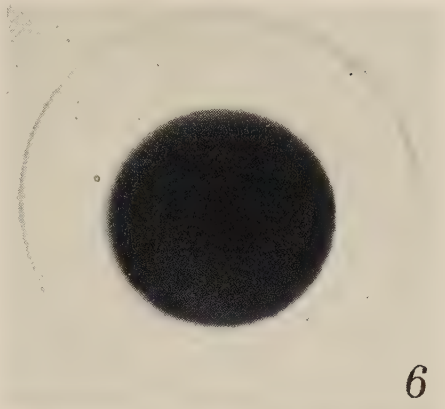
Electrical stimulation of eggs treated with Ca- and Mg-free artificial sea water or sodium oxalate.—Eggs were washed by Ca- and Mg-free artificial sea water or treated with sodium oxalate in order to find out whether, in the absence of Ca, membrane elevation followed electrical stimulation. Before treatment with direct current, the eggs were washed three times with Ca- and Mg-free artificial sea water and put in a capillary also containing Ca- and Mg-free artificial sea water. For the treatment with sodium oxalate, two or three drops of egg suspension in sea water were transferred to the oxalate solution and after three minutes were washed three times with Ca- and Mg-free artificial sea water and then into the capillary. A mixture of 0.2 ml of 0.2 *M* sodium oxalate and 0.8 ml of Ca- and Mg-free artificial sea water was used in these experiments.

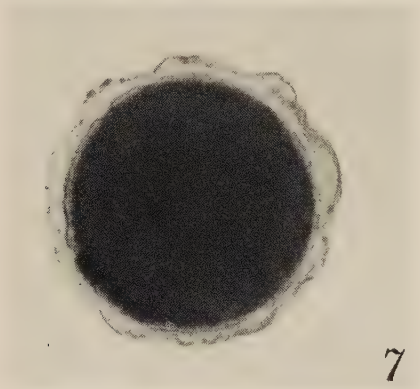
Fig. 5 shows the sequence of events. In these experiments membrane elevation occurred earlier at the cathodal side than at the anodal side and within a few seconds a complete membrane formed around the egg. Such membranes are higher and thinner than those of both normally fertilized and electrically stimulated eggs in sea water, and the perivitelline space is also wider and transparent. Except in the case when the membrane elevation stopped at the cathodal side (Fig. 5a) when the electrical stimulation was not strong enough, almost all eggs showed membrane elevation of concentric circular type similar to the egg of Fig. 5d and 6. Upon transferring such eggs to sea water, shrinkage of the membrane and formation of hyaline layer occurred (Fig. 7), and after a while monoaster appeared.

Electrical stimulation of urethane-treated eggs.—A 6 per cent solution of ethylurethane in sea water was used in these experiments. To check the fertilisability of the urethane-treated eggs at various time intervals the eggs were removed from the urethane solution, washed twice with sea water and then inseminated. When the eggs were treated with urethane for five minutes and then washed with sea water for ten or twenty minutes, insemination resulted in normal membrane formation and normal fertilization. Treatment longer than five minutes was followed by polyspermy and no membrane formation. Therefore, for the experiments of electrical stimulation, the eggs were treated with urethane for 10 minutes and then transferred to the capillary after being washed with Ca- and Mg-free artificial sea water.

On closing the electrical circuit, the membrane elevation took place at the cathodal side and the process followed the same pattern as in the eggs simply stimulated electrically in the absence of Ca, i.e. a thin membrane elevated which enclosed a wide perivitelline space. This, however, appeared to be filled with material extruded from the egg (Fig. 8), moreover the surface of the eggs was somewhat wrinkled. On the contrary in the case of eggs







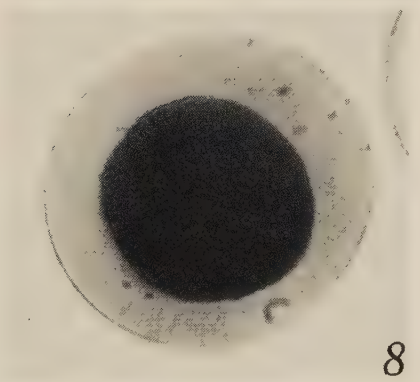


Fig. 5 (upper left). Process of membrane elevation of the egg stimulated in the absence of Ca.
Fig. 6. Egg stimulated in the absence of Ca.
Fig. 7. Shrinkage of membrane and hyaline layer formation of the egg of Fig. 6 transferred to sea water.
Fig. 8. Urethane-treated egg stimulated in the absence of Ca.

simply stimulated in the absence of Ca, such effects were rarely observed and eggs similar to that of Fig. 6 with wide and clear perivitelline space prevailed.

DISCUSSION

Electrical shock has been used as a method to induce a response from cells. As the physiological response of the egg to the proper stimulation is activation, it appeared quite interesting to investigate the type of response one would obtain as a response of an electrical shock. If, instead of an electrical shock, eggs are placed in an electrical field for a long period, some electrical change which happens during electrifying would be expected. For instance, movement of the eggs towards the certain pole and the membrane elevation of the egg during the closing of the circuit mean that some change of electrical resistance and subsequent change of electrical current take place. It is a serious problem either to calculate the potential gradient of electrical field or to keep egg material constant. For this reason the writer used the electrical shock which period is from $^1/_{20}$ to $^1/_{100}$ second.

The results obtained refer to the eggs of *Hemicentrotus*, but the cortical changes including the breakdown of the cortical granules and membrane elevation may possibly be applied to other species. The flattening of the cathodal side of the eggs, the rupture and extrusion of internal granules described by Moser [6] were only observed when strong cytolytic stimulations were applied. Therefore it is quite possible that the stimulations used by Moser were too long.

Electrical stimulation results in membrane elevation first at the anodal side and then at the cathodal side of the egg, and from there the membrane elevation spreads to cover the whole surface of the egg. This is certainly different from what occurs in the case of fertilization. However, if an adequate electrical stimulation is applied, the response from a certain part of the surface can propagate to the whole egg surface and breakdown of cortical granules and membrane elevation occur as in the response of fertilization. If on the other hand the applied potential gradient was too weak to induce complete membrane elevation, then the elevation occurred only on one side or both sides of the egg. This supports Sugiyama's contention [13] that the breakdown of one cortical granule at any point of the egg surface does not automatically induce the breakdown of its neighbours. If eggs with locally elevated membrane as a result of an electrical shock are fertilized, then breakdown of the cortical granules and membrane elevation all over the egg occurred, but the eggs become polyspermic. That shows that in these parts of the egg surface where no membrane elevation and no breakdown of the cortical granules occurred the polyspermy-preventing mechanisms had been abolished.

The perivitelline space of eggs electrically activated in sea water (Fig. 1, 2 and 4) is filled with cytoplasmic blisters. According to Motomura [7] the breakdown of the cortical granules is followed by the formation of a precipitation membrane at the inner surface of the vitelline membrane when it is elevated. Runnström, Monné and Wicklund [8] and Endo [2] showed also that the cortical granules merge with the vitelline membrane. It seems probable therefore that the electrical stimulation of the eggs in sea water can induce not only the breakdown of the cortical granules but also the outflow of some protoplasmic component. It appears furthermore that the surface change caused by an electrical shock does not propagate over the whole egg, if stimulation is done in sea water. On the contrary, when eggs are submitted to treatments such as washing away as much Ca as possible and then stimulated in Ca- and Mg-free artificial sea water, the surface change can apparently propagate to the whole egg surface: in fact the membrane elevated on both the anodal and the cathodal sides and a concentric circular type of membrane was formed. Therefore removal of Ca seems to be important for the propagation of the surface change. It seems interesting to recall here that a release of Ca following fertilization has been actually observed [4, 5].

Sugiyama [12] observed that urethane inhibited the propagation of the cortical response to some stimulating reagant. In the present work, the egg treated with urethane formed mainly an incomplete membrane even in the absence of Ca. The fact can be explained that the propagation of the response induced electrically may be also inhibited by urethane. The egg in Fig. 8 may have been submitted to a strong electrical shock so that the perivitelline space is not transparent and the egg about to undergo cytolysis. However, the difference of the difficulty of elevating an electrically clear membrane between the egg in sea water and the egg treated with urethane seems to be another matter. In the absence of Ca, the membrane elevation at the cathodal side followed by the elevation at the anodal side may be considered to have been induced by the changes of electric kinetic potential or another factor due to the changes in the external conditions and on the egg cortex itself.

Yamamoto [14] activated the ripe unfertilized egg of the fish, *Oryzias latipes* with direct current and observed the breakdown of the cortical alveoli embedded in the cortical protoplasm. Then elevation of the chorion began at the anodal and at the cathodal sides. The results presented in this paper support the interpretation [13] that the cortical granules of the sea urchin play the same role in membrane elevation as the cortical alveoli of *Oryzias*.

SUMMARY

1. Eggs of *Hemicentrotus* (*Strongylocentrotus*) *pulcherrimus* were subjected to an electrical shock with direct current which induced partial cytolysis.

2. In eggs stimulated with weak shock in sea water the membrane elevated at the anodal side or both at the anodal and the cathodal sides. When inseminated such eggs became polyspermic. If the electrical shock was adequate, either elliptical or spherical membrane with opaque perivitelline space was formed.

3. In the absence of Ca either washed away by Ca- and Mg-free artificial sea water or removed by sodium oxalate, the eggs elevated high and thin concentric circular membranes with transparent perivitelline space. These membranes shrunk upon transferring the eggs to sea water and the formation of a hyaline layer and of a monoaster took place.

4. Eggs treated with urethane elevated partial membrane at the cathodal side or both the cathodal and the anodal sides upon electrical stimulation.

5. The propagation of the response induced electrically is discussed on the evidence of the experimental results.

I wish to thank Prof. T. Yamamoto for suggesting and directing this investigation, and Dr. M. Sugiyama and Dr. E. Nakano for their stimulating discussions during the course of this experiment. My thanks are also due Prof. A. Monroy for his advice and for correcting this manuscript.

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STUDIES IN THE CORTICAL DARK FIELD COLOR CHANGE OF THE SEA URCHIN EGG

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Runnström [17] found that the surface of the ripe unfertilized sea urchin egg appears yellow-orange in dark field illumination. Upon fertilization the yellow color is rapidly changed into silvery-white or bluish-white. In a following paper [18] Runnström, using the dark field illumination, very thoroughly investigated the fertilization in several sea urchin species. Lindahl [9] corroborated these results and introduced as an experimental condition centrifugation of the egg. By using this method he was able to observe the dark field color change of the *Arbacia* egg and to study the fine structure of the egg cortex. A modified dark field technique was later used by Rothschild and Swann [16]. These authors correlated the dark field color change with the block to polyspermy.

The present paper gives an account of results obtained by using the phase microscope and the same dark field technique as that of Runnström.

MATERIAL

This work was carried out at Kristinebergs Zoological Station on the Swedish west coast, during June–August 1954. Two species of sea urchins were employed: *Echinocardium cordatum* and *Psammechinus miliaris*. The observations were made with a phase equipment from C. Reichert Optical Works, Vienna. The dark field illumination was provided by a "Zeiss Wechselkondensor nach Siedentopf" using homogenous oil immersion.

RESULTS

The influence of the precipitation of the jelly.—At Kristineberg sea urchin females containing eggs with precipitated jelly layers are fairly frequent. Despite this the eggs are easily fertilized and show normal development. Eggs with precipitated jellies were examined in the dark field microscope and the cell surface proved to have a distinct white color. Also the precipi-

tated jellies appeared opalescent white in dark field. In a few eggs the jellies were non-precipitated and these eggs exhibited the normal yellow color. Some of the eggs had jellies that were partly precipitated. In the non-precipitated part the surface color was yellow and in the precipitated the surface color was completely white. The cortical granules were intact and the eggs did not show any obvious signs of activation.

The occurrence of the white surface color in the eggs with precipitated jelly layers suggested experiments in order to determine if precipitation of the jelly is always correlated with white dark field color.

The influence of the JePptF(E) on the surface color.—In extracts from sea urchin eggs a factor has been found which precipitates the jelly layer of the intact egg [19, 23]. This factor, identical with the "antifertilizin" of Lillie [8], has been the aim of several investigations [7, 13]. Runnström and his school [7] adopted the descriptive term "jelly precipitating factor" (JePptF(E)) instead of antifertilizin, and this term will be used throughout this paper.

Unfertilized jelly-free eggs of *Psammechinus miliaris* were repeatedly washed in isotonic NaCl solution. The eggs were allowed to settle and the overstanding liquid was discarded. The concentrated egg suspension was two times sucked through a Pyrex G2 glass filter; this procedure resulted in a cell homogenate which was centrifuged for 30 minutes at 12,000 × g. All the operations mentioned were performed in a cold room at +4°C. The supernatant, which was free from particles visible in the microscope, was used as the source of JePptF(E).

JePptF(E) and normal unfertilized eggs in sea water were mixed in the proportion 1:25. The eggs were examined in dark field illumination and in the course of 30–180 seconds the yellow-orange cortical color disappeared. Also in eggs without jellies the color changed into bluish-white. The white color of the egg surface looked exactly like that of oocytes and fertilized eggs. The eggs were observed for about 30 minutes but no cortical breakdown seemed to occur and no membranes were formed. The precipitated jellies were also white in dark field illumination.

It has been demonstrated that treatment with antifertilizin from the sperm induces activation of the egg [22]. The jelly precipitating factor used in the present work, however, did not show any activating properties.

The influence of clupein on the surface color.—In another series of experiments clupein was employed to obtain precipitation of the jelly coat.

It has been demonstrated that clupein exerts a strong activating action on the sea urchin egg, indicated by the formation of low granular membrane, nuclear movement and cytoplasmic segmentation [24]. Upon insemination

the clupein treated eggs show poor membranes and heavy polyspermy. Clupein interferes with the breakdown of the cortical granules and therefore the growth of the hyaline layer is impeded resulting in polyspermic fertilization [4].

A 0.5 per cent clupein solution was prepared and the pH adjusted to 7.8–8.0. In the experiments unfertilized eggs in sea water and this stock solution were mixed to give a final clupein concentration of 0.001–0.05 per cent. The jelly coat became strongly precipitated and the egg surface immediately changed color from yellow-orange to white. Eggs lacking jellies also showed the same color change. The cortical granules were extruded to a high extent though no cortical breakdown could be stated. A thin hyaline layer was formed within the course of 2–5 minutes and this layer was found to contain the cortical granules and other small granules ($\sim 0.3\,\mu$), the latter granules appearing black in phase contrast. The hyaline layer was wrinkled and never reached the thickness of a normal layer formed subsequent to fertilization. In the clupein concentrations used membranes occurred very sparingly.

In oocytes a hyaline layer was described by Runnström and Monné [20]. They also showed that the cortex lacked cortical granules and that these granules were present in the interior of the oocyte near the nuclear membrane. Furthermore it was demonstrated that during the maturation process the granules migrate towards the cell surface. The oocytes always fail to give a fertilization membrane upon insemination and this fact clearly indicates the importance of the cortical granules for membrane formation. The hyaline layer does not grow which demonstrates that the cortical breakdown is essential for this process [4].

Also in ripe unfertilized eggs a thin (~ 0.3-$0.5\,\mu$) hyaline layer can be observed with good immersion objectives and in dark field illumination. This hyaline layer is readily preserved in fixatives containing mercuric chloride. The color of the layer is distinctly yellow-orange in dark field. Whether this hyaline layer constitutes the very surface of the egg or not is difficult to determine since much evidence has been produced about the existence of a vitelline membrane [20, 21, 22, 25]. Therefore it must be assumed that the vitelline membrane is submicroscopic if it covers the hyaline layer.

The effect of proteolytic enzymes.—When inseminated, eggs pretreated with proteolytic enzymes do not form fertilization membranes [5, 21). In order to study the effect of these enzymes on the color of the egg surface, eggs were treated with crystalline trypsin (Armour Co.), and crystalline chymotrypsin

(Worthington Biochem. Lab.), and with crude trypsin (Merck, Darmstadt). The crystalline enzymes were applied in a concentration of 0.002 per cent and the Merck-trypsin in a concentration of 0.01 per cent. In none of the experiments was the yellow dark field color changed into white. In Merck-trypsin, however, the color disappeared gradually and turned into pale yellow. Upon fertilization the normal white color appeared. The eggs treated with crystalline trypsin and Merck-trypsin showed fluffy hyaline layers with cortical granules lying on the egg surface [5, 25]. In chymotrypsin-treated material, on the contrary, the cortical granules seemed to behave in a normal manner. One gets the impression of a membrane formation without subsequent membrane elevation. The hyaline layer was also quite normal and no cortical granules were seen outside of the egg surface. This different action of trypsin as compared with chymotrypsin has been studied in fixed and stained material where the differences are as clear as in living material.

The action of lanthanum nitrate.—In another series of experiments the effect of $La(NO_3)_3 \cdot 6\ H_2O$ was studied.

A concentration of $10^{-2}M$ caused a very strong precipitation of the jelly coat. In dark field illumination the surface of the egg looked completely white. Following this treatment it was difficult to bring about fertilization which indicates a severe injury to the egg. Lower concentrations were employed but already in $10^{-4}M$ no effect on the dark field color was obtained.

The effect of nicotine treatment.—Rothschild recently investigated the action of nicotine on sea urchin fertilization [15] and found that nicotine acts by activating the egg and inducing polyspermy. The nicotine-problem has been discussed elsewhere [3] and in the present paper only its influence on the dark field color will be considered.

Unfertilized eggs were treated with nicotine in concentrations ranging from 1/500 to 1/1000 (volume/volume). After about 5–10 minutes the yellow-orange color weakened and turned into pale yellow. The egg cortex became swollen and it was observed that a few cortical granules broke down. 20–30 minutes after the treatment had started, some of the eggs (i.e., less than 10 per cent) showed membrane elevation. In the stronger concentrations the nicotine-effect was more pronounced but in none of the experiments a definite color change from orange to white was obtained. In the course of 1–2 hours a few eggs began to cleave and these eggs appeared white in dark field. If eggs which had been in contact with nicotine for 5–10 minutes were inseminated (concentration of sperm: 3.5×10^6/ml) they showed the normal color change to white. Many eggs (15 30 per cent) became polyspermic following such treatment.

DISCUSSION

The chemical composition of the egg surface has been widely discussed, and it appears that lipo-proteins and phosphatides constitute an important part of the surface layers [14]. The egg cortex is also very rich in carbohydrate and there may exist complex-compounds of lipids and carbohydrates [11, 12]. Such compounds are very unstable [2] which fact could explain why the light scattering properties of the egg surface are so easily changed. However, it must be considered that the pigment granules might also influence the dark field color of the egg (cf. [9]).

The block to polyspermy has been correlated with the cortical propagation and the development of the peripheral white dark field color [15, 16]. Rothschild and Swann [16] have also utilized the dark field change in their attempt to make a quantitative study of the probability of a successful sperm-egg collision. Furthermore they referred to the fact known by previous work [20] that oocytes, when inseminated, did not show any cortical propagation or membrane formation. The experiments with oocytes [20] indicate the role of the cortical granules. The oocytes are unable to propagate a block to polyspermy; this probably depends upon the lack of cortical granules in the cell surface. According to the results reported in this paper it is difficult to correlate the surface color change with the block to polyspermy. The jelly precipitating factor changes the yellow color into white and the eggs do not become polyspermic subsequent to fertilization. The same is observed after treating the eggs with lanthanum nitrate.

With clupein there is also a definite change of the dark field color, but in this case polyspermy is induced. Here the mechanism causing polyspermy seems to be clear; the cortical granules are precipitated by clupein resulting in poor membrane formation and impeded growth of the hyaline layer [4, 24].

The conclusion must be that the color change observed in dark field illumination is of no importance for the establishment of the block to polyspermy. This block is mainly related to the breakdown of the cortical granules and the subsequent growth of the hyaline layer [1, 3, 4, 5]. Since it has been shown that the dark field color change can be obtained without ensuing cortical breakdown, it seems preferable to follow the cortical propagation by direct observations of the cortical granules (cf. [1]).

Clupein and $La(NO_3)_3$ have one distinctive feature in common: a strongly basic character. The JePptF(E) must also be rich in basic groups and therefore it might be assumed that these substances react with the acidic phos-

phatides and/or carbohydrates of the egg surface thus bringing about the visible color change.

It has been shown that the JePptF(E) enhances the fertilization rate in intact eggs with jellies as well as in jelly-free eggs [6]. The strongly acidic jelly interacts with the JePptF(E) which is likely to contain basic groups. The egg cortex and the jelly are probably of the same composition [11, 12] and it seems evident that the promoting action of the JePptF(E) must be referred to a depolarisation of the jelly and the egg surface. The color change facilitates fertilization and therefore it might be regarded as reflecting a pre-activation of the egg.

It has been pointed out that the results obtained by treating the eggs with lipid solvents do not necessarily prove that the changes which appear refer to a dissolution of lipids for they might just as well be due to a denaturation of proteins [10].

The experiments with trypsin and chymotrypsin showed that the structures effecting the yellow-orange surface color stand this treatment. This negative result does not support the view that proteins are of special importance to the dark field color change.

SUMMARY

JePptF(E), clupein and $La(NO_3)_3$ change the dark field color of the unfertilized egg from yellow into white.

Jelly-free eggs show the same color change when treated with these substances.

Treatment with proteolytic enzymes or nicotine do not effect any distinct dark field color change.

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RECONSTITUTIVE EVENTS IN *BLEPHARISMA UNDULANS* AS AFFECTED BY COLCHICINE[1]

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It has long been assumed that the relationship between processes of visible cytologic structural differentiation and nuclear function is an intimate one, and that changes in one might be expected to affect the other. The drastic and profound effects of enucleation have been extensively studied in many cell types [22, 5] and evidence has accumulated that most processes that require synthetic mobilizations by the cell can be eliminated by enucleation or, again, restored by replacement with a new nucleus [6]. The striking effects of enucleation have led many to assign a "dominant" or "controlling" role to the nucleus in the nuclear-cytoplasmic complex [5, 19].

On the other hand, observations on enucleated cells or fragments indicate cytoplasmic activities, e.g. peptidase, that are apparently, at least temporarily, independent of the nucleus [14]. These activities are considered to be primarily of the "non-synthetic" type and involved in catabolic reactions of the cell. Other cytoplasmic components such as mitochondria, microsomes, chloroplasts, and basal granules of protozoa, in some instances, appear to have a fairly high degree of autonomy.

This study is a second of a series of studies that were undertaken to attempt to clarify relationships between the nucleus and cytoplasm, and to attempt to determine nuclear dependent activities, cytoplasmic dependent activities and those activities that are dependent upon both [13].

A system of cellular phenomena was desired that has obvious cytoplasmic structure and that has been shown to be "under nuclear control". Such a process is that of ciliate protozoan regeneration.

The processes of regeneration in *Blepharisma undulans* a large, pink, heterotrichous ciliate have been extensively studied [24, 30, 31]. An intimate association between regeneration and nuclear state has been described by Weisz [31]. A mechanism of ciliary redifferentiation has been worked out

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for other forms by Lwoff [20] and extended by Weisz [30] to *Blepharisma*. Regeneration is initiated by basal granular movement into the amputated area in a definite sequence so that the removed basal granules and cilia are replaced by the granules behind them in the ciliary row. New cilia, or associated structures are formed from the posterior replacing basal granules [20]. Weisz has attempted to show that the success or failure of regeneration in *Blepharisma* and *Stentor* (a closely related ciliate) is based upon the "physiological state" of the macronucleus [30].

In the protozoa, as distinct from the metazoa, regeneration of a fragment does not necessitate cell division, but rather the "reworking" of existing cytoplasm to produce, generally, a smaller replica of the damaged protozoan. In fact, removal of a certain amount of cytoplasm may abolish nuclear and cytoplasmic division [12] by apparently upsetting a critical ratio of cytoplasmic-nuclear volume.

The abolition of nuclear or cell division without immediate cell destruction can also be accomplished by critical dosages of certain physical agents, e.g. X-rays, ultraviolet irradiation or by such drugs as colchicine or other chemical agents. Depending upon the degree of nuclear damage produced by such agents, the effects may resemble those produced by enucleation. In the case of *Blepharisma*, enucleation of the entire organism is extremely difficult due to the complex macronuclear arrangement (see Fig. 1). Similarly it is most difficult to be certain that cut fragments are enucleate.

As was shown in the previous paper, the effects of ultraviolet irradiation could not, with certainty, be referred to either nuclear or cytoplasmic damage but rather appeared to involve both. The process of fission was shown to be more sensitive to the same wave lengths that delayed regeneration [13]. It was desired for the purpose of this study to select an agent, colchicine, that had been shown to primarily affect nuclear processes [6].

The effects of colchicine have been studied in a variety of forms, plant and animal, with astonishingly uniform results in those instances where the studies have been on the inhibition of the mitotic mechanism, and in cases where spindle formation has been shown to take place [18]. See also Eigsti [10] for the most recent bibliography on colchicine. Since in the ciliates only the micronuclei divide mitotically, the use of colchicine should abolish or inhibit micronuclear division, but may or need not affect the amitotic separation of the macronucleus. Exposure to colchicine of regenerating fragments possessing both types of nuclei may yield information as to the effects of colchicine on the process of regeneration and the possible involvement of mitosis with regeneration.

By exposing conjugants to colchicine, effects approaching enucleation might be obtained since it may prove possible to upset the micronuclear sequence in conjugation and produce a condition where there would be no new synkaryon to replace the disintegrating old macronucleus. Conjugant animals exposed to colchicine could then be examined and compared to enucleate fragments as to survival, respiration, etc. Also the effects on repigmentation and maintenance of minute fragments (possibly lacking any nuclear material) could be studied with the viewpoint of attempting to determine if there might be some underlying effect of colchicine that would account for the "toxic" effect at higher concentrations.

METHODS

The strain of *Blepharisma undulans* used in these studies was initially obtained from stocks maintained in the Biology Department, Washington Square College. Progeny from a single isolation were used in experiments. Mass clonal cultures were grown at room temperature (about 20°C) in lettuce medium inoculated with *Pseudomonas ovalis*, shown to be a suitable food organism [13]. Organisms to be used for the various studies were taken from the test tubes about one week after the initial inoculation and thus about one week prior to conjugation, which usually occurred on or about the 14th day after inoculation. Cannibal-giants were obtained by first enlarging individuals by previous feeding with *Chilmonas* and *Khawkinea*. The individuals so enlarged then commence feeding upon smaller *Blepharisma*. Giants so produced were more alike in size, than those produced in dwindling cultures.

Studies of the effects of colchicine upon regeneration of the anterior half of the animal, including the peristomal field and the cytostome, but excluding if possible the macronucleus, were designed. The cytostome of the animal was removed by a single slice across the end of the peristomal field (see B, Fig. 1). Groups of animals were cut in either various concentrations of colchicine or in Brandwein solution, in deep cell depression slides coated with agar, under a low power dissecting microscope. For the studies of smaller fragments, cuts were made at A, Fig. 1, and the anterior tip retained.

All individuals used in any of the series came from batches of 50 individuals washed at least four times in sterile, buffered (pH 7.0) Brandwein solution before being treated. Isolations subsequent to washing and cutting were made in each of the spots of a 12-spot Klein agglutination slide containing a thin layer of agar and the desired concentration of colchicine diluted with Brandwein solution or in in the case of the controls, sterile Brandwein solution. Checks of pH of all of the solutions were frequently made. On a given agglutination slide the entire group of 12 isolations were individuals that had received the same treatment. In all experiments the agglutination slides were maintained in moist chambers at room temperature in a dark closet.

After the desired time of exposure, e.g. 6 hours, 24 hours, was reached, each organism was isolated into a depression on an agglutination slide containing a thin layer of agar and lettuce medium previously inoculated with *Pseudomonas ovalis*.

When pigment-free animals were desired, they were bleached at room temperature under a 60-watt lamp for 24 hours prior to use in cutting or whole animal studies. The light was passed through a water bath to reduce the effects of heat.

Conjugants were obtained by the method of Young [32] by placing a clone into an embryological dish in sterile Brandwein solution without additional food for 24 to 48 hours. Cutting of conjugant pairs was performed, as above, again using a single transverse cut across the two joined animals. The single cut separated the conjugant pair into three fragments; an anterior fragment containing portions of both conjugants fused at the peristomal area, and two posterior fragments from each of the conjugants.

Isolated individuals were maintained in sterile Brandwein solution or desired colchicine-Brandwein concentrations for 6 hours, 24 hours, or one week. Excepting for the groups exposed for the one week period the remainder were transferred into lettuce-*Pseudomonas* medium as described above, at the end of the 6 hour or 24 hour exposure.

In early exploratory experiments, concentrations of colchicine-Brandwein solution from 10^{-2} *M* to 10^{-11} *M* were employed to determine the more critical dosages. These were found to be between 10^{-3} *M* and 10^{-6} *M*. Lysis of the *Blepharisma* oc-

TABLE I

Fission. Whole *Blepharisma*.

Description of group	No. animals in group	Molarity	First fission delay	Third fission delay	Mortality	Recovery
I. Normal pink 6 hours	36	10^{-3}	S	NS	S	C
II. Normal pink 24 hours	36	10^{-3}	S	S	S	C
III. Normal pink. Permanent in colchicine	24	10^{-3}	?	—	S	—
IV. Cannibal pink 6 hours	24	10^{-3}	NS	NS	NS	C
V. Cannibal pink 24 hours	12	10^{-3}	NS	NS	NS	C
VI. Cannibal pink. Permanent in colchicine	24	10^{-3}	NS	S	S	I

TABLE II

Regeneration. Posterior portions cut in colchicine.

Description of group	No. animals in group	Molarity	Regeneration delay	First fission delay	Third fission delay	Mortality	Recovery
I. Normal pink 6 hours	60	10^{-4}	S	NS	NS	?	C
	60	10^{-3}	S	NS	NS	S	C
II. Normal pink[1] 6 hours	12	10^{-4}	S	NS	NS	NS	C
	12	10^{-3}	S	NS	NS	NS	C
III. Normal pink 24 hours	12	10^{-4}	?	NS	NS	NS	C
	12	10^{-3}	S	S	S	S	I
IV. Normal bleached 6 hours	12	10^{-3}	S	NS	NS	NS	C
V. Cannibal pink 6 hours	24	10^{-4}	?	NS	NS	NS	C
	24	10^{-3}	S	NS	S	NS	C

[1] Cut in Brandwein.

S = Significant (P less than 0.05).
? = Questionable (P near 0.05).
NS = Not significant (P greather than 0.05).
C = Complete recovery (more than three fissions).
I = Incomplete recovery (never reached three fissions).

curred within minutes of exposure to 0.1 M, making the use of this concentration impractical because of possible osmotic effects. Concentrations lower than 10^{-6} M produced effects that were indistinguishable from the controls in all cases.

The number of animals in the entire series of experiments was not large since each one had to be hand manipulated to a considerable degree and observations had to be made over a long period of time thereafter. In most cases, at least three different series were performed, except where otherwise noted. (See Tables I, II, and III.) What was lost by lack of larger numbers was made up in the knowledge of the behavior of each individual in the course of all the treatments. The general uniformity of performance was tested by measures of deviation and tests of significance for small samples [28].

As discussed above the following groups were studied in greater or lesser detail.

(1) Effects of colchicine 10^{-3} to 10^{-6} Molar on fission rate of pink, normal, whole *Blepharisma.*

(2) Effects of colchicine 10^{-3} to 10^{-6} Molar on fission rate of whole, pink, cannibal-giant *Blepharisma.*

Table III

Regeneration and fission in pink conjugants.

Description of group	No. animals in group	Molarity	Separation delay	Regeneration delay	First fission delay	Third fission delay	Mortality	Recovery
I. Whole pairs 6 hours	12	10^{-3}	NS	—	NS	S	S	I
II. Whole pairs 24 hours	12	10^{-3}	S	—	S	S	S	I
III. Whole pairs Permanent in colchicine	12	10^{-3}	NS	—	Died prior to first fission	Died	S	All died
IV. Anterior portions of pairs 6 hours	12	10^{-4}	S	S	?	S	NS	AC
	12	10^{-3}	S	S	?	S	NS	AC
V. Separated posterior portions of pairs,6hours	12	10^{-4}	—	S	?	NS	S	C
	12	10^{-3}	—	S	S	S	S	C

AC = Almost complete.
(Others as in Table I).

(3) Effects of colchicine 10^{-3} to 10^{-6} Molar on fission rate of whole, bleached, normal *Blepharisma*.

(4) Effects of colchicine 10^{-3} to 10^{-6} Molar on regeneration of pink, normal *Blepharisma*.

(5) Effects of colchicine 10^{-3} to 10^{-6} Molar on regeneration of pink, cannibal-giant *Blepharisma*.

(6) Effects of colchicine 10^{-3} to 10^{-6} Molar on regeneration of bleached, normal *Blepharisma*.

(7) Effects of colchicine 10^{-3} to 10^{-6} Molar on pink, normal conjugant fragments.

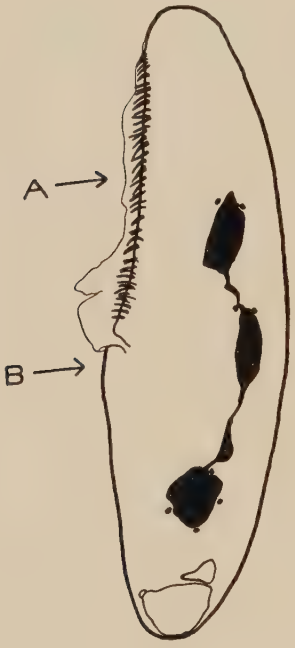
(8) Effects of colchicine 10^{-3} to 10^{-6} Molar on pink, normal, small anterior fragments.

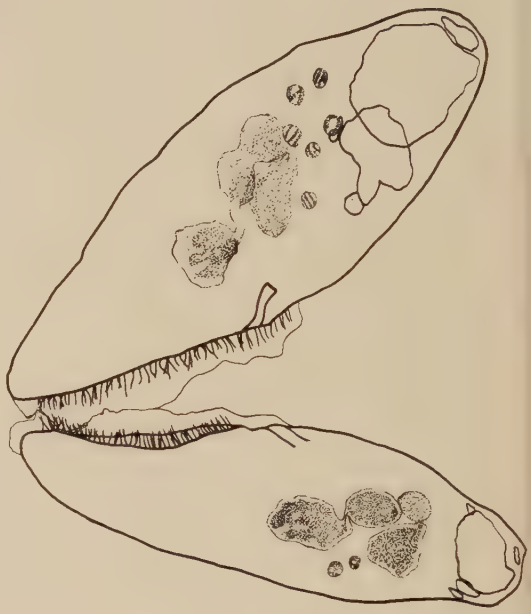
Most of the above groups were carried out in 6 hours, 24 hours, and 1 to 2 week exposures.

In interpreting the tables, the term "Significant" is based on a probability analysis between control groups and experimental groups for each of the series where the probability is less than 0.05 [28]. "Normal" individuals refer to non-cannibal-giant forms. "Pink" refers to unbleached forms. "Recovery" is based on surviving animals if they ultimately each undergo at least three divisions, even though they may exhibit delays. The criteria for regeneration are the reconstitution of the anterior portion of the animal especially the cytostomal and peristomal fields.

The effects of colchicine on the food organism, *Pseudomonas ovalis*, were tested

with weighed quantities of colchicine added to the cooling nutrient agar to produce final concentrations of 10^{-3} and 10^{-4} Molar. Control tubes lacking colchicine were similarly prepared. The tubes were incubated at 37°C.





All of the following figures were taken from Feulgen preparations. × 970.
Fig. 1 (upper left). Whole mount of normal pink *Blepharisma undulans*, camera lucida, showing peristomal membranelles, a four lobed macronucleus and eight micronuclei. The organisms were cut at *"A"* for small fragments and at *"B"* for regeneration studies.
Fig. 2 (upper right). Conjugating normal *Blepharisma undulans*, camera lucida, showing macronucleus and dividing micronuclei.

RESULTS

The data on the effects of colchicine on some of the processes studied are summarized in Tables I, II and III. A significant delay in reaching the first three fissions was produced by 24 hour exposure to 10^{-3} *M* in the normal whole animals and posterior fragments. A 6 hour exposure to 10^{-3} *M* significantly delayed only the first fission in the normal pink group. After the initial delays the fission rate in the exposed groups was comparable to the controls, i.e. recovery. Lower concentrations, e.g. 10^{-4} *M*, did not produce fission delay in the same groups affected by the higher concentrations. See also Tables I and II.

Figs. 3 and 4 compare a control *Blepharisma* in fission and an experimental organism exposed for 24 hours to 10^{-3} *M* colchicine. In the control organism, Fig. 3, the micronuclei have finished their mitotic cycle and are in inter-

Fig. 3 (lower left). Normal *Blepharisma undulans* in division. Note condition of the micronuclei. Fig. 4 (lower right). 24 hour exposure to 10^{-3} *M* colchicine, camera lucida, note cytoplasmic irregularities and peristomal field, also mitotic figures of micronuclei although this is a later stage than Fig. 3.

phase while the macronucleus and cytoplasm have just begun to divide. In the colchicine treated animal, Fig. 4, the macronucleus and cytoplasm are in a much later stage of division, but the micronuclei show the typical colchicine effect, a suspension of mitosis. Note however, that the cytoplasm and macronucleus are continuing to divide.

Uncut cannibal-giant forms showed sensitivity to colchicine only when permanently maintained in 10^{-3} *M*. Posterior fragments of cannibal-giants showed a delay in reaching a third fission in 10^{-3} *M* at 6 hour exposure.

The whole animals in conjugation and fragments of conjugants exhibited on the whole, a greater sensitivity to colchicine than the non-conjugating

animals. In some groups recovery after removal from colchicine did not occur. See Table III.

Regeneration of the anterior tip was delayed in some groups by 6 hour exposure to 10^{-4} *M* colchicine and in all groups by 6 hour exposure to 10^{-3} *M*. Subsequent fission of these cut forms was comparable to that in the control groups. See Table II.

The survival of many of the groups studied was affected by 10^{-3} *M* among the whole animals, conjugants, conjugant and non-conjugant fragments. Again the cannibal-giant forms appeared less sensitive, only those permanently maintained in colchicine showed a higher death rate than their controls. Small anterior tip fragments, possibly lacking nuclear material, showed a higher death rate when exposed for 6 hours to 10^{-3} *M* colchicine than did the unexposed fragments.

All of the bleached forms exposed to colchicine regained their pigmentation as rapidly as did the unexposed bleached controls. Conjugation was observed after recovery in the exposed groups at about the same time as their controls. No effects of colchicine on *Pseudomonas ovalis* were noted.

DISCUSSION

Effects of lower concentrations of colchicine (10^{-4} M and below) on fission rate.—The data obtained in these studies indicate that lower concentrations of colchicine have little or no effect in delaying cytoplasmic division in *Blepharisma*. This lack of effect of lower concentrations has been described for some other organisms, e.g. *Paramecium* spp. [7, 11, 17], bacteria [26], yeast [27]. In contrast, higher plants and animals show a greater sensitivity to low concentrations since fission is delayed in chick embryonic tissue cultures by 4×10^{-7} *M* [15], embryonic grasshopper neuroblast by 2.5×10^{-6} *M* [15], hydra-tentacle regeneration by 10^{-5} *M* [29], *Arbacia* eggs by 2×10^{-4} *M* [2] and staminal hairs of *Tradescantia* by 10^{-4} *M* [25].

There is an apparent cytological difference between the response of the micronuclei and macronuclei of *Blepharisma* to colchicine (Figs. 7 and 8). The macronuclei of both the control organism (Fig. 8) and of the experimental (Fig. 7) appear to be unaffected, whereas mitosis in the micronuclei of the experimental organism appears to be disrupted by the 24 hour exposure to 10^{-4} *M* colchicine. The cytological data further indicate that this disruption of the micronuclei has little or no effect on cytoplasmic division (Figs. 3 and 4), whereas in higher plants and animals such a disruption of mitosis would have completely suppressed cytoplasmic division [15]. The

P. 422, "Discussion", line 12, 13, 14. *Figs. 7 and 8* should be Figs. 5 and 6, respectively.

cytological and fission data on *Blepharisma* indicate that in the ciliate protozoa, where there is an amitotically dividing nucleus, the macronucleus, the division of the cytoplasm is probably unaffected by disruptions of the mitotically dividing micronuclei.

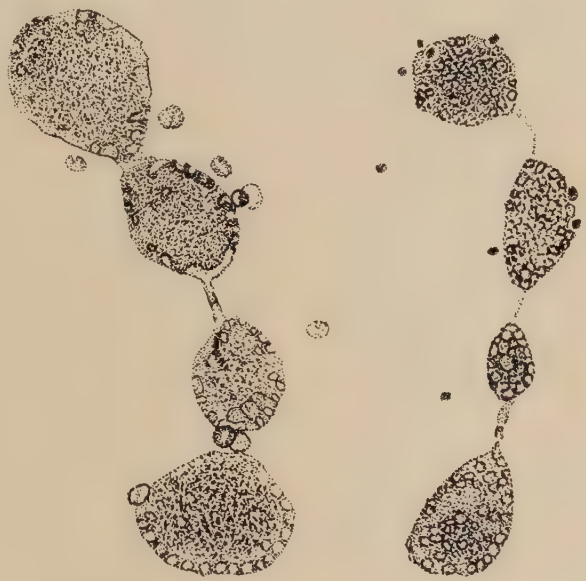


Fig. 5 (left). 24 hour exposure to 10^{-4} *M* colchicine. Camera lucida drawing. Note that all eight micronuclei show "swelling".
Fig. 6 (right). Control for above. Note normal appearance of the nine micronuclei.

Another possible factor in the lack of effect of 10^{-4} *M* on fission delay is the non-synchronous division of the micronuclei ([32], also our own observations) of *Blepharisma*. Thus at any one time, only a few of the many (up to 14) micronuclei undergo mitosis, followed by macronuclear division and cytoplasmic division. Colchicine apparently inhibits those micronuclei at metaphase, while the division of the cytoplasm places a number of the interphase micronuclei into each daughter cell.

The role of the micronuclei in fission is thus not settled, but results obtained in this study indicate the relative unimportance of the micronuclei in cytoplasmic division. The interrelationship between regeneration and the nuclear apparatus in *Blepharisma* is more affected by the macronuclear condition than the micronuclear condition as studies at 10^{-4} *M* indicate.

Cytological observations of Feulgen preparations of colchicine treated cannibal-giants by 10^{-3} *M* for 24 hours and the untreated controls showed the presence in both groups of a *c*-mitotic effect on the micronuclei. This may be interpreted that there is a polyploid condition normally present in

the cannibal-giant group. This is further substantiated by the macronuclear apparatuses that have at least two more lobes than the non-cannibal-giant *Blepharisma*. It may be possible to conclude that the change from normal to cannibal-giant involves the production of a polyploid condition as exhibited by a more lobed macronucleus and polyploid micronuclei.

Effects of lower concentrations of colchicine (10^{-4} M and below) on regeneration.—The observations on regeneration sensitivity to colchicine at lower concentrations indicate a wider area of action of colchicine than previously reported. To explain the results on regeneration, the critical observations of Inoué [16] on the effects of colchicine on the spindles of dividing *Chaetopterus* eggs, can be applied. Inoué showed a depolarization effect of colchicine on the anisotropic fibers of the spindle at 5×10^{-4} M. A system of subpellicular polarized fibers have been described by Metz [23] in *Paramecium*, lying between and attached to the basal granules and forming part of the typical ciliate neuromotor fibrillar system. A mechanism of ciliate regeneration has been worked out by Lwoff [20]. According to Lwoff, regeneration depends upon a basal granular (kinetosome) movement into the amputated area in a definite sequence so that there is a replacement of the removed cilia and the granules by new outgrowths of the basal granules of the same ciliary row that have migrated into the area.

The results obtained in this study suggest that colchicine may disrupt the anisotropic fibrillar systems in *Blepharisma* so that there may be produced a dislocation of the migration of the basal granules. This would effectively block reconstitution of the amputated area and its associated structures.

A plasma membrane or cortical effect by colchicine, is, however, also possible and might be responsible for the delay. In addition, the possibilities of a primary macronuclear effect that would produce a secondary delaying effect on regeneration cannot be ignored, although the last seems less likely in view of the lack of effect of the same concentration on division delay, a process that might be expected to be more closely associated with macronuclear function than regeneration.

The first paper on ultraviolet effects on *Blepharisma* [13] showed regeneration less affected by critical wave lengths, than cytoplasmic division, particularly those wave lengths strongly absorbed by nucleic acids and proteins. The present study indicates a greater sensitivity of regeneration to colchicine than cytoplasmic division. The processes of regeneration may necessitate the utilization of cytoplasmic elements, primarily the oriented protein fibers that are more sensitive to colchicine and less sensitive to ultraviolet than the proteins in the cytoplasm needed for cytoplasmic division.

Effects of colchicine on whole conjugant pairs and conjugant fragments.— The importance of the macronucleus in the existence of ciliate protozoa is shown by the effectiveness of lower concentrations (10^{-4} *M*) of colchicine on conjugant fragments and conjugant whole animals. In other cells studied, unless death or lysis of the cell occurs colchicine effects are reversible if the treated organisms are returned to a normal environment. The recovery of treated forms similarly takes place in *Blepharisma*, except in the conjugant groups.

In *Blepharisma undulans*, as in many of the ciliates, the macronucleus degenerates after conjugation and is totally replaced by derivatives from the synkaryon nucleus [31, 32]. Thus any total micronuclear disruption by colchicine treatment during conjugation might produce a condition where no new macronucleus could be produced. Irreversible effects of colchicine, leading to death before three divisions were produced in all of the conjugant groups except in the posterior fragments. The complete recovery of the surviving posterior fragments might be explained on the basis that the plane of cutting would leave most of the nuclear material behind in the posterior fragments (Fig. 2).

The recovery of the posterior fragments might then be due to micronuclear reorganizational processes, e.g. selfing, or possibly by the retention of the old macronucleus or parts of the old macronucleus.

A similar explanation may apply to the lack of effect of lower concentrations of colchicine on the fission on the bacterium *Pseudomonas ovalis*, and on other lower organisms where the division of the cell may involve a multinucleate system [8] or amitotic separation of the chromatin material. Another possible explanation is that certain of the lower organisms such as yeast or bacteria may be able to utilize colchicine as nitrogen source [26, 27]. The same explanation cannot be ruled out for the protozoa. In a mixed culture containing both bacteria and protozoa, exposures of even very high concentrations of colchicine (10^{-2} *M*) may be ineffective for a variety of reasons.

The possibility of colchicine acting as a nitrogen source for bacteria may explain some of the stimulatory effects on protozoa obtained by exposing mixed cultures containing bacteria. The results would then be primarily an augmented bacterial nitrogen source that would result in an increasing number of food organisms and a decreasing available amount of colchicine to affect the protozoa. This would contribute to an increased protozoan fission rate that might be erroneously interpreted as a direct stimulatory effect of the colchicine on the protozoa. This entire problem raised by at-

tempts to obtain precise effects of a chemical agent in mixed cultures was thoroughly tested and reported on by Ball [1].

Effects of higher concentrations of colchicine (10^{-3} M and above) on fission, regeneration, and fragment longevity.—The effects of 10^{-3} *M* colchicine on *Blepharisma* indicate a widespread colchicine effect that culminates at 0.1 *M* in a characteristic lysis of the organism. These effects might correspond to the "toxic" or "lethal" effects reported on higher organisms and probably indicate a disruption of one and possibly more of the critical organizational systems of the organisms. Since polarized structures are colchicine sensitive, one of the drastic effects may be due to plasma membrane or cortical disturbances or destruction. The recent work of Mazia and Dan [21] indicate that the sulfhydryl linkages are important in spindle formation. The interesting hypothesis of Dustin [9] is that the spindle sensitivity to colchicine may in reality be a sulfhydryl effect and the toxic or lytic effect could then be thought of as a wide-spread sulfhydryl effect. On the other hand, the recent work of pyrophosphatase inhibition by Bloch-Frankenthal and Bach [4] and inositol oxidase by Benitez *et al.* [3] indicates that colchicine effects *in vivo* at high concentrations may be relatively non-specific.

SUMMARY

1. The effects of selected dosages of colchicine (10^{-2} to 10^{-13} *M*) were studied in a red-pigmented, heterotrichous ciliate *Blepharisma undulans* on the following: (1) division, (2) regeneration of cut *Blepharisma*, (3) conjugation, (4) survival of whole *Blepharisma*, regenerating and non-regenerating fragments.

2. Concentrations below 10^{-4} *M* were ineffective on all processes studied. Higher concentrations (10^{-2} *M* and above) were cytolytic to *Blepharisma*.

3. Reconstruction of the red pigment in bleached forms was unaffected by colchicine. The response of bleached forms to colchicine was the same as unbleached organisms. Cannibal-giants were less affected by colchicine on the processes studied than normal forms.

4. The effect on division indicated that in ciliate protozoa where there is an amitotically dividing nucleus, the macronucleus, the division of the cytoplasm is apparently unaffected by disruption of the mitotically dividing micronuclei. 10^{-4} *M* produced micronuclear mitotic inhibition without affecting cytoplasmic division, 10^{-3} *M* affected both processes.

5. Conjugating whole *Blepharisma* or conjugant fragments were more affected by lower concentrations (10^{-4} *M*) than non-conjugating forms. In

some instances conjugating organisms exposed during conjugation did not recover from colchicine exposure. The inability to recover may possibly be due to a disruption of the new nucleus.

6. The process of regeneration in *Blepharisma* was more affected by lower concentrations (10^{-4} *M*) than was division. The effect on regeneration may possibly be due to a disruption of the ciliary fibrillar systems.

7. An explanation is offered for the change from the normal-sized individual to the cannibal-giant. This change may be due to the production of a polyploid individual.

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A METHOD FOR MEASUREMENT OF THE ABSORPTION IN EXTREMELY HIGH-ABSORBING SOLUTIONS

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Spectrographic determination of the concentration of light-absorbing solutions presupposes knowledge of the range in which Lambert-Beer's law is valid and also of possible deviations from this law. In highly concentrated solutions there are often deviations from the linear relation concentration–extinction. It is most unusual that deviations occur from the linear relation thickness of layer-extinction (often called Lambert's law). In biologic ultramicrospectrography there appear concentrations of absorbing substances of up to 20 or 30 per cent, in several cases yielding more than 99 per cent absorption for a layer thickness of only $10\,\mu$. The essential difficulties in examining the range of validity of the aforementioned law in model experiments are partly to be found in the manipulation of the highly concentrated solutions, partly in the production of a cuvette, the thickness of which is sufficiently accurately known when it lies in the range of $1–20\,\mu$. Moreover, solutions which are usually highly viscous should be easy to apply to the cuvette.

Fig. 1 shows a device, a "lens-cuvette", which overcomes these difficulties. A plane-convex lens is placed with its convex surface downwards on a plane-parallel glass (or quartz) plate, and the solution is applied between them. Since the distance between the lens and the plane surface varies from zero upwards, the most suitable thickness can be selected during the measurement. The thickness of the layer at the measuring point is determined from the distance between the point and the optical axis of the lens, which passes through the point of contact where the thickness should be zero. The convex lens surface is spherical with the radius $=R$. The distance from the measuring point to the optical axis $=x$ and the thickness $=t$.

$$x^2 = t\,(2\,R - t) \tag{1}$$

But $t \ll R$

$$\therefore\ t = x^2/2\,R. \tag{2}$$

From (2) it may be seen that t (and thus also the transmission) varies very slowly with x in the immediate vicinity of the optical axis. Therefore it is not convenient to determine the position of the optical axis by transmission measurements. Avoiding this difficulty the determination of x can be carried out in the two following ways.

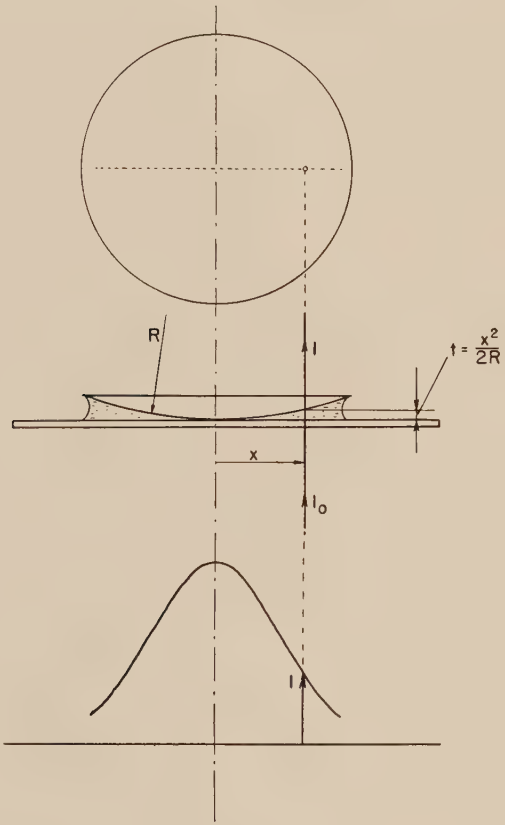

Fig. 1. A lens-cuvette for measurement of the absorption in extremely high-absorbing solutions.

I. This is based upon the condition that Lambert's law is valid. The plane plate with the lens and the solution is placed on the object stage of a microscope. The microscope, equipped with a dry system, projects an image of the plane in which the liquid is situated on to a photocell placed at a distance of 2 m. There a diaphragm cuts out a small area of the image corresponding to a measuring area of a few μ^2 in the cuvette. The measuring spot is selected by movement of the object stage. Its position may be seen on a micrometer screw. The transmission or only the intensity of the transmitted

light is measured at three or more points along a straight line through the optical axis. Transmitted light is expressed: I_0, I_1, I_2, I_3, ..., and the distance from the optical axis: x_0, x_0+x_1, x_0+x_2, x_0+x_3, ..., where x_1, x_2, x_3, ... can be measured exactly, while x_0 is unknown and is determined from Lambert's law:

$$\varepsilon = \frac{e_{10}}{t_1 - t_0} = \frac{e_{20}}{t_2 - t_0} \tag{3}$$

$$e_{10} = {}^{10}\log (I_0/I_1), \qquad e_{20} = {}^{10}\log (I_0/I_2) \tag{4}$$

$$t_0 = x_0^2/2R, \qquad t_1 = (x_0+x_1)^2/2R, \qquad t_2 = (x_0+x_2)^2/2R \tag{5}$$

From this we obtain

$$x_0 = (x_2^2 - k \cdot x_1^2)/2(k \cdot x_1 - x_2) \tag{6}$$

$$k = e_{20}/e_{10}.$$

This value for x_0 is inserted in (3) which gives the desired value of the extinction coefficient ε. For higher accuracy x_0 (and ε, of course) should be calculated as a mean value for a number of different combinations according to (3).

II. In this case the whole continuous transmission curve is determined for a line going through the optical axis of the lens. As the transmission curve is symmetric in relation to the optical axis, its position can be determined very accurately. The principle equipment for the measurements is similar to that in case I and consists of the ultramicrospectrophotometer constructed at this institute. For details on the measuring apparatus see [1, 2, 3, 4]. The object holder, which is a stage especially designed for this purpose, is carried by steel springs instead of quartz plates, making it possible to move it 4–5 mm. It is moved by a precision micrometer screw connected to a reversible motor via a system of selsyn motors. The measuring data (the transmission) are registered on an XY-recorder. The paper in the XY-recorder is moved synchronously with the object stage. Fig. 2 shows an original curve for a 4 per cent RNA solution measured at a wave length of 2650 Å. A 1 mm movement of the paper corresponds to approximately 16 μ at the object. The total movement of the object holder with the "lens-cuvette" is about 4 mm., i.e. the measuring line through the optical axis is 4 mm in length (cf. Fig. 3 in [4]).

On the basis of this transmission curve the thickness of the layer is calculated for a number of points as $t = a^2/8R$ where $a = 2x$. The corresponding $e_t = {}^{10}\log (I_0/I)$ is calculated where I_0 is an arbitrary value, and e_t is plotted as a function of t. If Lambert's law is valid, this curve should be a straight line with the slope giving the extinction coefficient ε. (Cf. Fig. 3.) Moreover,

it is of no importance whether or not there is true contact at the optical axis between the lens and the plate, as the variation in the thickness of the layer and not its absolute value is utilized in the calculation of ε.

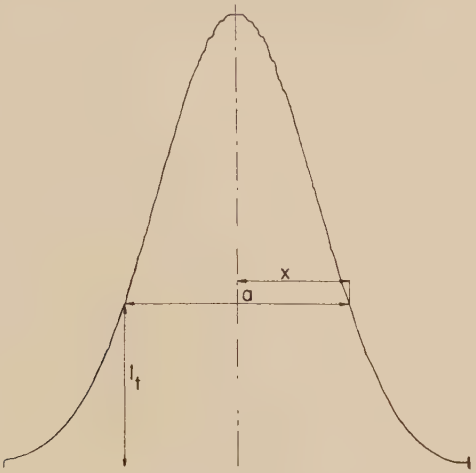

Fig. 2. An original curve for a 4 per cent RNA solution measured at a wave length of 2650 Å.

The relation of the paper movement to the movement of the object stage is examined by means of an object micrometer scale on the object stage. This examination was done with and without the lens on the scale. In both cases relation between the movement of the paper and that of the object was linear to within ± 0.2 per cent.

It may be difficult to adjust the object table in such a way that the measuring line goes through the optical axis, but it is facilitated by Newton's rings, which are visible before the solution is applied. The optical axis passes through the center of the rings. When the measuring line intersects the optical axis, the upper boundary of the liquid layer consists of a great circle on the sphere of which the convex surface of the lens is a part (2). The radius of a great circle = that of the sphere. If the measuring line does not intersect the optical axis, the measuring beam is in a plane parallel to the optical axis. Consequently, the upper boundary of the liquid is still a circle as an intersectional line between a plane, and a sphere always consists of a circle. This circle, however, is less than a great circle, as every other circle on a sphere is less than a great circle of the sphere. If the distance between the line and the optical axis is z, the radius of the circle is r.

$$r = \sqrt{R^2 - z^2}$$

But $z < R$

$$\therefore r = R(1 - z^2/2R^2).$$

In our case where $R = 100$ mm the error $z^2/2R^2 < 0.01$ per cent when $z < 2$ mm. It is evidently not absolutely necessary to adjust the measuring line so as to pass through the optical axis. It is easy to obtain $z < 0.1$ mm. That the thickness of the layer is $z^2/2R$ at the top of the curve is of no importance, as seen above.

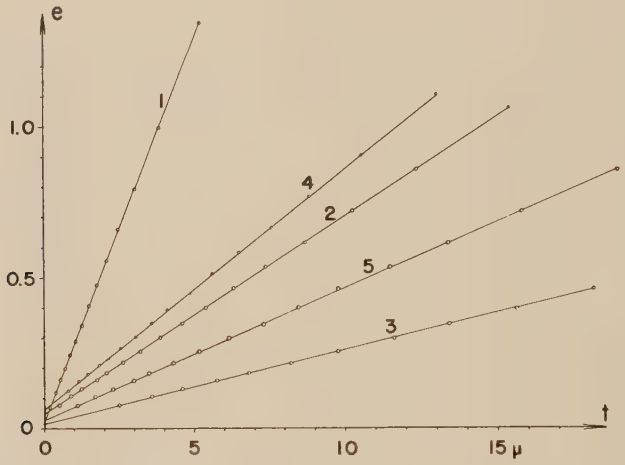

Fig. 3. $e = {}^{10}\log (I_0/I)$, t = the thickness of the layer.

Curve 1:	Xylene	2650 Å,	$\varepsilon = 0.256 \pm 0.001$
„ 2:	Picric acid	3660 Å,	$\varepsilon = 0.0655 \pm 0.0001$
„ 3:	Naphthol in cyclohexanone . . .	3223 Å,	$\varepsilon = 0.0244 \pm 0.0001$
„ 4:	4 per cent RNA solution	2650 Å,	$\varepsilon = 0.0808 \pm 0.0004$
„ 5:	4 per cent RNA solution	2800 Å,	$\varepsilon = 0.0439 \pm 0.0002$

The fact that the area of the measuring spot is finitely large implies that the measured value of the transmitted light consists of the mean value for the area. In no case is the diameter of the measuring spot greater than 20 μ. Even under extremely unfavorable conditions 20 μ would not cause an error greater than 0.1 per cent. Therefore this source of error may be ignored.

Whether or not the lens has any influence on the measuring area was investigated by measuring a non-absorbing substance, water at 5461 Å. The transmission was constant along the whole line. This shows that the lens does not change the measuring area but that, on the contrary, it is constant along the whole measuring line.

Fig. 3 shows the result of a number of measurements. Table I corresponds to the two curves with the greatest and the least slope. The curves as well as Table I show that Lambert's law is valid for the solutions examined.

TABLE I

Xylene 2650 Å			Naphtol in Cyclohexanone 3223 Å		
$t\mu$	e	ε	$t\mu$	e	ε
0.21	0.125		2.50	0.126	
0.32	0.153		3.57	0.155	
0.41	0.177		4.60	0.179	
0.54	0.211		5.75	0.208	
0.70	0.248		6.80	0.232	
0.86	0.292		8.20	0.265	0.0246
1.05	0.338		9.75	0.303	0.0246
1.27	0.390	0.250	11.60	0.347	0.0239
1.49	0.455	0.258	13.40	0.393	0.0243
1.75	0.524	0.261	15.60	0.445	0.0241
2.08	0.605	0.257	18.10	0.509	0.0245
2.46	0.708	0.259	20.30	0.579	0.0249
3.02	0.841	0.255			
3.82	1.043	0.253			
5.17	1.387	0.255			
Mean value		0.256			0.0244
		± 0.001			± 0.0001

SUMMARY

A method is described for the measurement of the absorptions in extremely high-absorbing solutions. The cuvette consists of a plane-convex lens on a plane plate. The measurements can be carried out either point by point in a microscope or by continuous recording in e.g. the ultramicrospectrophotometer of this Institute. Measurement may be carried out in ultraviolet as well as in visible light. The thickness of the layer varies from a small fraction of a micron to 30 or 40 microns, making it possible to determine ε within an approximate range of 0.01–5 per micron. The accuracy is better than ± 1 per cent and, in favorable cases, may be considerably better, as seen from the examples given.

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A RECORDING FUNCTION TRANSFORMER AND INTEGRATOR FOR MICROSPECTROGRAPHIC USE

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Curves giving the light transmission along a measuring line traversing a microscopic object are obtained with the recording ultramicrospectrophotometers [1, 2, 4, 5]. These measurements are carried out at certain definite wave lengths, so that one transmission curve along the measuring line is obtained for each wave length. By locating the measuring line so that it is representative of the entire object measured (e.g. a cell) or by traversing several parallel measuring lines at equal intervals covering the entire object, measured data are obtained on the light transmission in different parts of an inhomogeneous object [cf. 2]. These transmission data are subsequently used for calculation of the total quantity of absorbing substance in the object. For this it is also necessary that the magnitude of the object and the absorption coefficient of the substance be known.

The first step in the calculation is to transform the transmission curves so that curves are obtained in which the ordinate is proportional to the quantity of absorbing substance, i.e. the extinction (ε) is computed, defined by

$$\varepsilon = -^{10}\log T, \tag{1}$$

where T is the transmission (the transmitted light intensity divided by the incident light intensity). In order to obtain the total quantity of absorbing substance in the object these extinction curves must then be integrated, and the integral must be multiplied by a factor which takes into consideration the magnitude of the object, the number of measuring lines and the extinction coefficient of the substance.

To permit statistical treatment of the reliability of the measurements the number of objects measured must be large. As a result the transformation and integration of the transmission curves becomes so time-consuming and routine an operation that it has been considered justifiable to develop some kind of automatic computer for the purpose. In order also to permit transformation of similar photometric curves obtained in measurements using the interference microscope [3] and photometric curves from microradiograms [7] it was considered necessary to design the apparatus in a manner

so that the transformation of the transmission curves can take place according to an arbitrary function. Moreover, it should be possible to connect the apparatus to the microspectrograph for transformation and integration in conjunction with the measurement as well as to utilize it for treatment of previously recorded curves [see 2, 6].

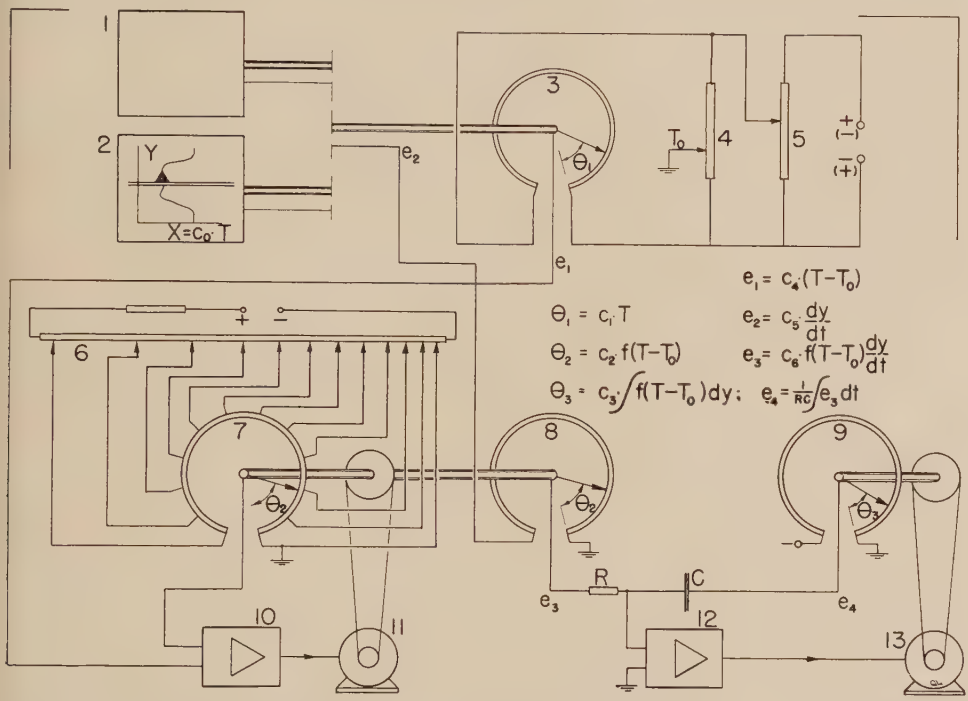

Fig. 1. Principle of function-transformer and integrator. *1*, recorder for light-transmission; *2*, handoperated curve-follower; *3*, *4*, *5*, potentiometers for transmission (*3*), zero adjustment (*4*) and span adjustment (*5*); *6*, resistance wire with adjustable taps; *7*, potentiometer with equally spaced taps; *8*, *9*, potentiometers in the X_1–X_2 recorder; *10*, *12*, servo amplifiers; *11*, *13*, servo motors.

In the choice between an analog computer and a digital computer it was from several points of view found advantageous at this stage to select an analog computer. In the first place it has been considered necessary to have continuous recording in the form of a curve of both the transformed transmission curve and the integral. This is most easily achieved with an analog computer. In the second, the accuracy attainable with an analog computer is quite sufficient. Finally, it is less complicated and costly than a digital computer. A brief report on the principles for the apparatus has been published earlier [8].

THE FUNCTION TRANSFORMER

A "Speedomax" X_1–X_2 redorder is used to plot the results. It is a self-balancing and recording potentiometer with two measuring circuits, each with its own recording pen. The pens move across the paper parallel with each other, approximately only one millimeter apart. One of the measuring circuits is used to plot the transformed curve and the other to record the integral of the former curve (see below).

The function transformation takes place in an electric analogy circuit with a non-linear potentiometer (Fig. 1). The transmission values can be fed into the instrument in the form of an angle of turn (θ_1 in Fig. 1), obtained from a manual curve tracer. This angle of turn is applied to a potentiometer, *3*, which delivers a voltage proportional to θ_1. The instrument can also be directly coupled to the ultramicrospectrophotometer, in which case the transmission values are given by the potentiometer, *3*, located in the transmission recorder of the microspectrophotometer. From the voltage, e_1, it is possible with the aid of potentiometer *4* to subtract a voltage proportional to the light transmission (T_0) around the preparation (free area). The potential then obtained from potentiometer *3* can be expressed:

$$e_1 = c_2 (T - T_0). \tag{2}$$

The constant, c_2, may be varied with potentiometer *5*, which supplies current to potentiometers *3* and *4* connected in parallel.

The voltage, e_1, is measured with the non-linear potentiometer combination, *6*–*7*. *7* is included as the measuring potentiometer in one of the two measuring circuits in the X_1–X_2 recorder. It is relatively high-ohmic (100,000 ohms), and has been equipped with a number of contacts along the winding. These lie at equal intervals and are electrically connected with individual movable contacts on the low-ohmic potentiometer, *6*. As *7* is very high-ohmic, its load on *6* is negligible. Thereby it is possible to assume that the different contacts receive the voltages corresponding to their positions on the linear potentiometer, *6*. By suitable adjustment of the contacts on *6* it is possible to make the relation between the potential difference and the angle of turn for potentiometer *7* follow the desired transformation function. However, it does not follow the transformation exactly, but with a linear approximation between the different points of contact.

The procedure followed in the transformation is shown schematically in Fig. 2, where the ordinates in both coordinate systems represent voltages,

while the abscissas correspond to angles of turn or deflections of the recorder. System A makes it possible to visualize the translation of the transmission deflection into a voltage with the help of a linear function. The adjustment of the position of the line is determined by two values on the abscissa, e.g. T_0 and 0.1 T_0, and is carried out with the aid of potentiometers *4* and *5*

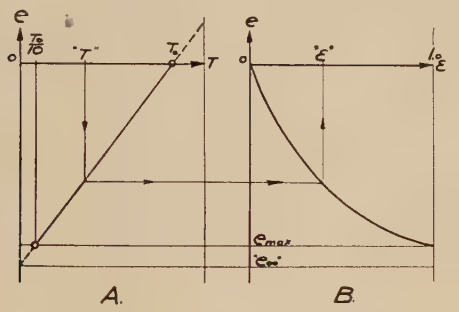

Fig. 2.

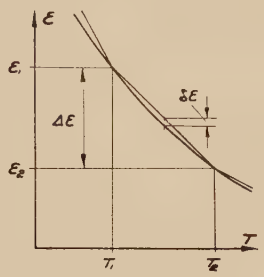

Fig. 3.

Fig. 2. Principle for transformation of transmission (T) to extinction (ε).
Fig. 3. The error in the function-transformer.

(Fig. 1) in such a manner that the voltage, e_1, becomes zero and e_{max}, respectively (Fig. 2.). The latter voltage corresponds to deflection *1* (e.g. full deflection) on the measuring circuit. This is represented by coordinate system B in Fig. 2. The voltage obtained from A is measured here, and the deflection of the recorder follows the preselected function (the curve in B). The measurement is carried out in the ordinary manner by one servo circuit of the recorder (*10* and *11* in Fig. 1), which sets potentiometer *7* at the voltage e_1.

Because the transformation curve is approximated with a series of linear segments with the ends located on the curve, there is an error which is greatest at about the middle of such a segment, and zero at the ends (Fig. 3). In the case where the transformation curve consists of the relation between transmission and extinction,

$$\varepsilon = -{}^{10}\log T, \tag{1}$$

and the projections on the ε-axis are of equal length (i.e. equidistant contacts on potentiometer *7* in Fig. 1), the maximum error expressed in extinction becomes equally large for all segments. An investigation of the magnitude of the maximum error shows that it is

$$\delta\varepsilon = \frac{\Delta\varepsilon}{10^{\Delta\varepsilon}-1} - {}^{10}\log e + {}^{10}\log \frac{10^{\Delta\varepsilon}-1}{\Delta\varepsilon \cdot \ln 10}, \tag{3}$$

where $\delta\varepsilon$ = the maximum error,

$\Delta\varepsilon$ = the length of the segment's projection in the ε-axis, and

e = the base of natural logarithms.

Potentiometer 7 (Fig. 1) is divided into 20 equal parts, of which, however only 18 can be used. If the apparatus is set to give full deflection for the extinction, 1.0, which is usually done, $\varepsilon = 1/18$. This gives a maximum error of

$$\varepsilon = 0.00089,$$

i.e. less than 0.1 per cent of full deflection. This accuracy is quite sufficient considering the degree of accuracy in the instrument as a whole. For example, the linearity tolerance for potentiometer *3*, which converts the transmission value to voltage, is given at 0.5 per cent.

THE INTEGRATOR

The integrator included in the apparatus is of the Miller type. The principles of its operation are evident from Fig. 1. The voltage, e_3, which is to be integrated, supplies current through the resistance, R, to the condenser, C, which is thereby charged. In order that the charging current shall be dependent only upon the voltage, e_3, and not be affected by the potential difference in the condenser, which, of course, rises with the charge, the point of connection between R and C is maintained at zero potential. This is achieved with the aid of a servo circuit (*9*, *12* and *13* in Fig. 1), which regulates the voltage, e_4, on the other side of the condenser. Due to this regulation, e_4 becomes exactly the voltage to which the condenser is charged. Since the charge of the condenser, Q, is proportional both to the product of the capacitance, C, and the voltage, e_4, and to the time integral of the charging current, $i = e_3/R$, the following relation is obtained

$$Q = -C \cdot e_4; \tag{4}$$

$$Q = \int_0^t i\,dt = \int_0^t \frac{e_3}{R}\,dt. \tag{5}$$

The minus sign in (4) occurs because e_4 must be negative if e_3 is positive and of the regulation is done so that the connecting point between R and C has zero potential.

(4) and (5) give

$$e_4 = -\frac{1}{RC}\int_0^t e_3\, dt. \tag{6}$$

Measuring potentiometer *9* and its recording pen will thus give a deflection that is proportional to the time integral of voltage e_3. This deflection is a measure of the integral of the transformed primary curve, since e_3 is taken from potentiometer *8*, which is turned synchronously with the function transforming potentiometer, 7. The integral is correct only on condition that the recording paper is allowed to run at a constant speed during the curve tracing so that dt in the integral (6) can be replaced by $k \cdot dy$ (i.e. $dy/dt =$ a constant). By y here is meant the coordinate parallel to the paper movement. The same requisite of constant rate of movement of the paper, and thereby of the preparation, exists also, of course, when the apparatus is directly connected with the microspectrophotometer. Inasmuch as the scanning speed can be changed, it is shown in Fig. 1 how this variable can be introduced into the integrator. Potentiometer 8 is fed with a voltage proportional to the scanning speed. The voltage e_3 will then assume the form of

$$e_3 = c_6 \cdot f(T - T_0) \cdot dy/dt, \tag{7}$$

i.e. it is a measure of the product of the transformed primary curve and the scanning rate. The deflection, θ_3, is then determined from the following integral:

$$\theta_3 = c_3 \int_{y_1}^{y_2} f(T - T_0)\, dy. \tag{8}$$

As may be seen, the integral now has the desired form since it does not have time but rather the scanning coordinate, y, as independent variable. c_3 is a constant factor which includes, among other elements, the magnitude of R and C in the integrator (Fig. 1) and proportionality factors for the relation between potentials and scale deflections.

The integrator gives a correct result only on condition that the leakage of condenser C is negligible and that the input impedance of the servo amplifier does not load the integration circuit. No loading occurs, however, if the servo circuit is in balance, since the input voltage is then zero. This assumes that the amplifier is sensitive and does not deliver any interfering signal, which is automatically compensated, of course, with an input potential that differs from zero.

The effect of leakage of condenser C is evident if we investigate the error

it causes in integration of a constant voltage during a certain period. Using the symbols in Fig. 1 we obtain

$$-C\frac{de_4}{dt}-\frac{e_4}{R_L}=\frac{e_3}{R}. \tag{9}$$

R_L is the resistance to leakage of the condenser. On condition that e_3 is constant and $e_4=0$ for the time $t=0$, the following solution to this differential equation is obtained:

$$e_4=-e_3\cdot\frac{R_L}{R}\cdot\left(1-e^{-\frac{t}{R_LC}}\right), \tag{10}$$

or developed to a series:

$$e_4=-\frac{e_3\cdot t}{RC}\cdot\left(1-\frac{t}{2R_LC}+\frac{t^2}{6(R_LC)^2}-\cdots\right). \tag{11}$$

The factor before the parenthesis is the correct integral value when e_3 is constant. The factor inside the parentheses is a correction factor that takes the leakage of the condenser into consideration. The condenser used in the apparatus has a leakage time constant of about $R_LC=5\times10^5$ seconds. The rate of the paper feed in the recorder is such that the integration time, t, is normally about 2 minutes. This gives the following value for the correction factor:

$$1-0.24\times10^{-3}.$$

Thus, the error in the integral due to the leakage of the condenser is less than 0.05 per cent in the most commonly occurring studies. As the result of a special coupling, which will be discussed in the following section, the error will not be greater even if the integration time is prolonged arbitrarily.

As mentioned above, the integration circuit is not loaded by the input impedance of the amplifier when the circuit is in balance. In the process of balancing or if the amplifier delivers an interfering signal, however, there will be an error in the integral due to the loading. In order to keep this at a minimum the amplifier must have great amplification, so that the imbalance voltage is negligible in comparison with the integrand voltage, e_3 (Fig. 1). Moreover, the input impedance of the amplifier must be great, so that the current taken out of the integration circuit in imbalance is negligible in comparison with the charging current, e_3/R. The first condition is easy to fulfill. The amplification must not be raised to such an extent, however, that oscilla-

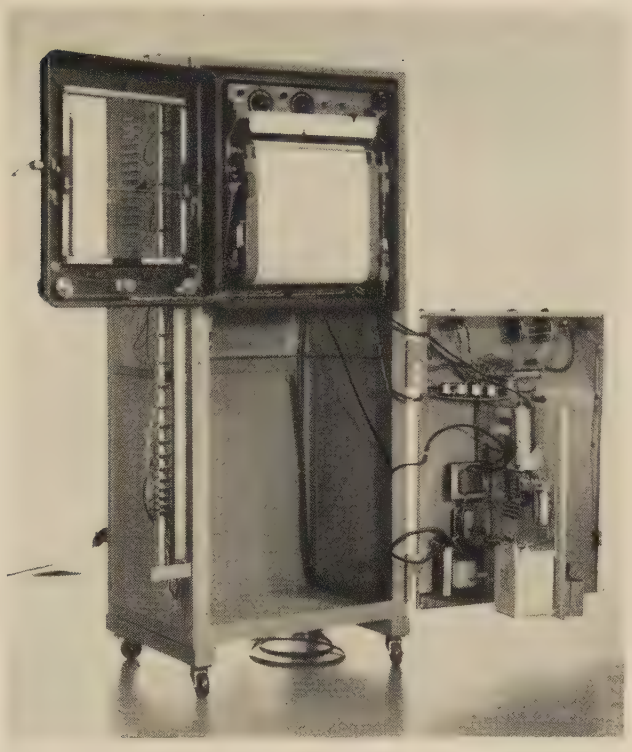

Fig. 4. View of the instrument with the doors opened to show the X_1–X_2 recorder (top) and the integrator (bottom).

tion occurs in the circuit. The second requirement has been satisfied by complementing and modifying the servo amplifier, *12* (Fig. 1), as explained in the following section.

PRACTICAL CONSTRUCTION

The apparatus is built into a cabinet. The upper part of this cabinet contains the X_1–X_2 recorder. The door of the lower part of the cabinet has the control panel on the outside and the chassis of the integrator on the inside. Thus the chassis is drawn out when the door is opened, making it easily accessible for inspection and repairs (Fig. 4). On the left side is potentiometer *6* (Fig. 1), which consists of a resistance wire stretched along a millimeter scale. The adjustable contacts lie along a rod beside the resistance wire. In front of the cabinet a removable table is attached (Fig. 5) to provide an arm rest for the operator, who follows the transmission curve on the moving recording paper with an indicator controlled by two knobs. Two knobs are used to permit the use of both hands and thus to attain more

Fig. 5. The instrument in operation with the operator tracing the transmission curve.

certain tracing of the curve. The knobs are connected with a ten-turn potentiometer (*3* in Fig. 1), which drives the indicator across the paper by means of a wire. This entire curve-tracing mechanism is built into the door of the recorder. On the control panel immediately under the recorder are the main circuit breaker, breakers for the integrator and the paper movement device, and knobs for the setting of the constants in the function transformer (c_2 and T_0 in equation (2)) and for the zero setting of the integrator.

The diagram of the apparatus is given in Fig. 6. However, the standard amplifier of the recorder is not included there. The apparatus is designed for coupling into the a.c. network, but the current for the conversion and integration circuits is obtained from dry batteries. The servo amplifier of the integrator has been complemented with an amplifier with high-ohmic input. The accompanying vibrator has been removed from the recorder and placed in the vicinity of the integrator circuit. In the standard recorder the

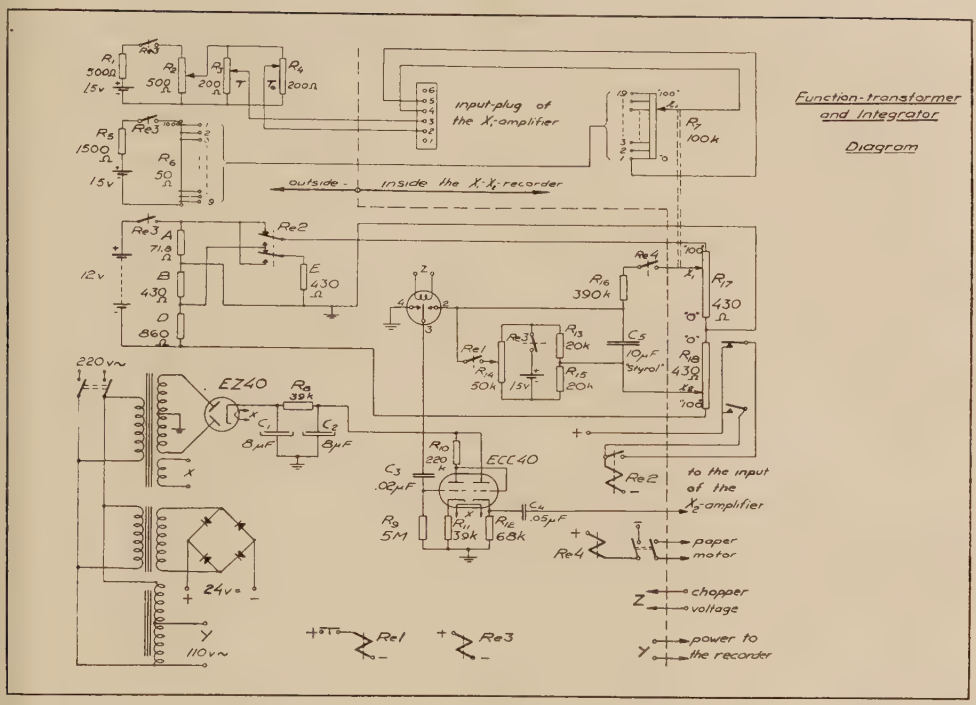

Fig. 6. Circuit diagram.

vibrator contacts are short-circuited during a small portion of the period. This results in a short-circuit which grounds and rapidly discharges the integration condenser, even if there is a minimum of imbalance voltage. By adjusting the vibrator so that the middle contact is connected with only one side contact at a time, this inconvenience is eliminated. With the coupling shown in the diagram the load on the integration circuit is at least twice the input resistance in the amplifier, i.e. 10 megohms, which has proved fully satisfactory.

The integrator is equipped with a mechanism that changes the sign of the integrand voltage (e_3 in Fig. 1) when the integral pen has reached either end position. Thereby the integration can continue indefinitely and independent of the limited width of the paper. When the integral pen moves to the right, e_3 is positive and the condenser is charged; when it moves to the left, e_3 is negative with discharge as a result. This device also affords another advantage. As mentioned earlier, the error due to the leakage of the condenser is appreciably reduced. During the period of charging the leakage causes a negative

error, while the error is positive during discharge, Accordingly, the resultant error becomes only the difference between these and may, in favorable cases, be zero.

The sign changing takes place with the help of end position contacts in the pen mechanism. These operate a relay (*Re* 2, Fig. 6), which permits the integrand potentiometer, R_{17}, to change positions with a resistance of equal magnitude (E) on the negative side, with respect to potential, of the group of resistances A, B, D, E, R_{17} and R_{18}. These resistances are of such dimensions that all current and voltage magnitudes remain unchanged by the sign change. Because of the coupling a common battery can be used for the integrand and the integral. The integration factor is therefore independent of the magnitude of the battery voltage. Resistances A, B, D and E are wire-wound and specially adapted for the purpose.

For the zero setting of the integrator there is a pressure button on the control panel, which operates a relay that short-circuits the integration condenser. This may also be given an initial voltage from potentiometer R_{14}. This is necessary because the electric and mechanical zero positions of the integrator do not agree exactly.

The function transforming potentiometer (R_7 in Fig. 6; 7 in Fig. 1) consists of a linear, wire wound potentiometer which is equipped with equidistant tappings on the winding. Since the potentiometer is high-ohmic and consequently wound with fine wire, it is difficult to devise these tappings. The problem has been solved through the use of small ball bearings as contacts. These are pressed against the outside of the winding. The winding is polished to permit good contact. The ball bearings are held in position by a bakelite ring that has holes for the ball bearings and surrounds the winding.

During direct connection to the microspectrophotometer the potentiometer for curve tracing (*3* in Fig. 1) is replaced by a potentiometer that is built into the photometer's Speedomax recorder. This recorder registers the light transmission, and the potentiometer thus gives a voltage proportional to the transmission. The device suggested earlier, which takes into consideration a variable scanning rate (voltage e_2 in Fig. 1) has not proved necessary. The scanning rate is constant and can be reproduced with an accuracy of approximately 0.5 per cent.

ACCURACY

The error of the function transformer has proved in practical tests to be less than ± 0.5 per cent of the full deflection, for transformations of the types met within biological ultramicrospectrographic work.

The accuracy of the integrator was investigated through determination of the constancy of the factor, k, for different values of the integrand, x_1, in the following expression of the relation between the two pen deflections in the X_1–X_2 recorder:

$$x_2 = k \cdot \int_{y_1}^{y_2} x_1 \, dy; \tag{12}$$

y_2–y_1 is the movement of the paper. This shows that k is constant within ± 1 per cent. It has a value of 0.020 if x_1 and x_2 are given a value of 1 at full deflection and y is measured in millimeters.

The value of the factor, k, changes with the automatic sign changing for the integrand by less than $^1/_4$ per cent.

Since its completion and testing the apparatus has been in constant hard routine use without the occurrence of any serious disturbances. It has been utilized principally for transformation and integration of transmission curves. The integrator has also proved useful for other purposes. It has been used in computation of the light distribution in interference rings as the distribution occurs if it is measured with a photocell with a diaphragm diameter that is not small in comparison with the width of the interference rings.

This paper is the major part of a thesis for the degree of a "civilingenjör" at the Division of Automatic Control of The Royal Institute of Technology, Stockholm, Sweden.

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STUDIES ON ISOLATED CELL COMPONENTS

VIII. HIGH RESOLUTION GRADIENT DIFFERENTIAL CENTRIFUGATION[1]

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The purpose of the studies described in this paper has been to lay the groundwork for the development of a technique for analytical medium-speed centrifugation in which quantitative separation of nuclei, whole cells, mitochondria, microsomes, soluble proteins, and lipid inclusions may be achieved in one or two steps. Since it is not possible, at present, to make quantitative measurements of all particulate fractions during centrifugation in a manner analogous to that used in the ultracentrifuge, it is necessary to rely on methods which allow fractions to be recovered, free of cross-contamination, for analysis at the end of the centrifugation. Fortunately, the procedures which are necessary to achieve convection-free sedimentation in suspensions of cell components are readily adapted to do this. The method to be described has been developed over a period of several years and is based in part on previous work done on starch gradients (N. G. Anderson, 1949, unpublished) and on techniques described by Behrens [1] and by Brakke [3, 4].

It appears not to have been generally realized that the separation of tissue brei constituents in a centrifugal field cannot be predicted from a simple uncritical application of Stokes' law. If one assumes, for example, equivalent radii of 4 μ for nuclei and 1 μ for mitochondria, their sedimentation velocities should differ by a factor of approximately 16, depending on the densities of the particles. Actually, no such clear-cut difference in sedimentation rate is observed, and a few nuclei are often found still in suspension after many of the mitochondria have sedimented. In methods wherein a brei layer is used over a denser clear layer of solution such as the one described by Hogeboom *et al.* [8], one observes mitochondrial contamination of sedimented nuclei even when many nuclei are still to be found in the upper layer. This and other anomalous effects may be shown to be caused by several artefacts which are common to nearly all isolation procedures.

[1] Work performed under Contract No. W-7405-eng-26 for the Atomic Energy Commission.

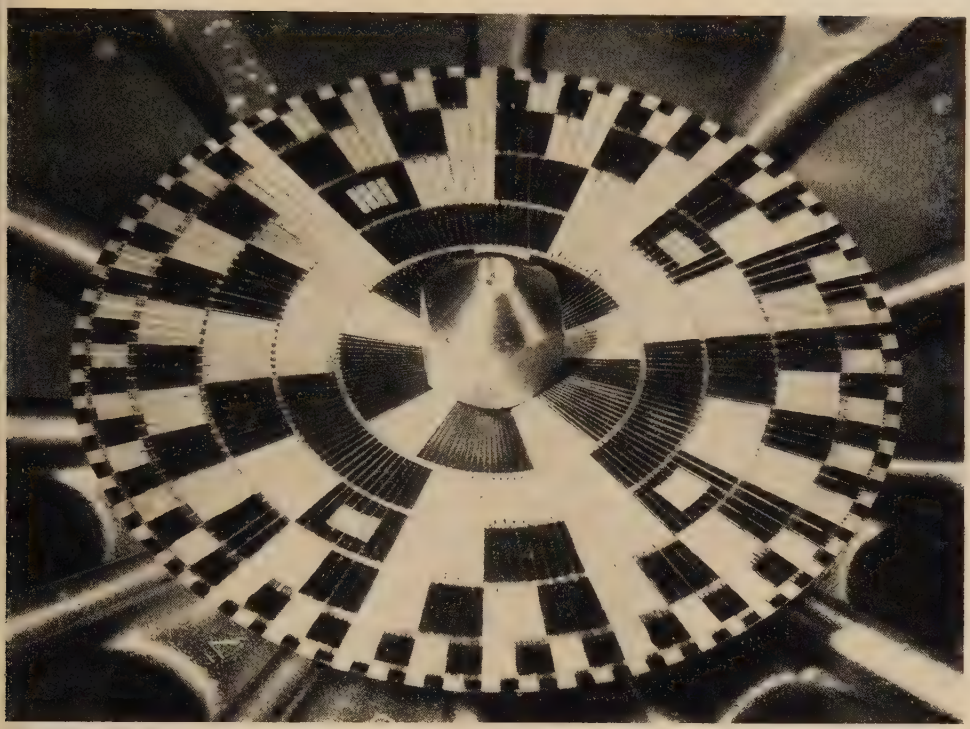


Fig. 1. Stroboscope disc for accurate speed determinations.

METHODS AND MATERIALS

In the present work two centrifuges were used: an International Equipment Company Size 2 centrifuge modified by the installation of refrigerating coils, suitable insulation, two observation ports on the hinged cover, and a stroboscopic lamp mounted inside the centrifuge; and, more recently, a specially modified Model PR-2 refrigerated centrifuge[1] from the same manufacturer. Alterations in the latter include installation of a large observation port of shatterproof glass, an electrical contactor[2] on the main shaft for continuous stroboscopic observation during sharp changes in speed, steel jacks to raise the centrifuge off the rubber castors to reduce vibration, and a stroboscopic lamp[3] mounted inside the centrifuge. By the use of a stroboscope[4] and an auxiliary high-intensity lamp[5] alone or in conjunction with the contactor described, a flexible system is obtained for the observation of centrifuge tubes at

[1] International Portable Refrigerated Centrifuge, Model PR-2, International Equipment Co., Boston, Mass.

[2] Contactor Type No. 1535A. General Radio Co., Cambridge, Mass.

[3] Strobotron, Type 631-Pl, manufactured by Sylvania Corp. for General Radio Co.

[4] Strobotac, Type 631-B, General Radio Co.

[5] Strobolux, Type 648-A, General Radio Co.

all speeds, during rapid changes in speed, and for the accurate (> 0.02 per cent) determination of speed, 60-cycle line frequency being used as standard. Cutout shields allow the centrifuge tubes to be observed during centrifugation. An aluminum disc (Fig. 1) with concentric rings divided into 144, 72, 36, 18, 6, and 10 divisions, alternately black and silver, is placed in the center of the centrifuge head for speed determinations with a lamp flashing frequency of 3600/minute. Speeds are read off at 50-rpm increments with a minimum of repetition of patterns, and 25 rpm increments (zero or hundreds plus 25 or 75) appear as clear lines in a grey outer ring. The speed increments observed with any set of black divisions is found by dividing the flash rate (3600) by the number of black divisions. Thus the disc described gives stable patterns for the various rings at multiples of 50, 100, 200, 400, 1200, and 720 rpm respectively reading from the outside in. Minor modifications such as placing silver centers in four divisions of the 400-rpm increment ring differentiates 400 rpm from 800 rpm. With head number 253 used in these experiments, a stable image of the head is also obtained at 300-rpm increments. The sequence of patterns obtained on acceleration and deceleration is readily memorized. To obtain accurate acceleration and deceleration curves, the times at which various speeds were reached were recorded on a printing chronograph.[1]

CENTRIFUGATION ARTEFACTS

Hydrodynamic effects.—Two types of hydrodynamic effects are seen. The first, here termed the *streaming effect*, occurs in very low gravitational fields and is in fact easily studied in stationary tubes. It does not appear to involve movement of the particles out of their original suspending medium. The second, termed the *turnover effect*, occurs during centrifugation and is observed in layered systems when the suspended particles pass an interface between solutions having different densities.

The streaming effect is seen when a 10 per cent liver brei is layered over sucrose solutions having a slightly higher density. As shown in Figs. 9–14, material soon begins to fall in streams from the brei layer through the denser layer *without any centrifugation*. With the systems described either by Wilbur and Anderson [15] or by Hogeboom *et al.* [8], much material reaches the bottom of the tube within 5 minutes. Microscopic examination shows that nuclei, whole cells, and mitochondria are all present in the sediment. In further studies, breis were prepared in Solution I [15] in such a manner as to contain 20, 10, 5, and $2\frac{1}{2}$ per cent rat liver by weight, and were layered over Solution III [15]. Decreasing anomalous sedimentation was seen with decreasing liver concentration, or with increasing density of the under layer (Fig. 9, 10). When a 10 per cent brei in Solution I was layered

[1] Tracergraph, No. SC-5A, modified to print consecutive times from starting time. Tracerlab, Inc., Boston, Mass.

over 0.88 *M* sucrose containing the same salts as found in the homogenizing medium, only a few small streams of descending material were seen, and these appeared only after an interval of about 30 minutes. Little qualitative difference was noted between tubes maintained at room temperature and those kept at 4° C during observation. Similar results were obtained with suspensions of rat erythrocytes (Figs. 12–14).

The streaming effect appears to be the result of local statistical fluctuations in the number of particles per unit volume which produce local concentrations of particles sufficiently high to increase the density above that of the lower fluid. The heavy particles (nuclei, whole cells, mitochondria) then begin to sediment as a group, dragging along the fluid between them and associated smaller particles. As the droplet moves through the denser fluid, diffusion equalizes the sucrose concentration.

The *turnover effect* occurs in layered systems when particles migrate through the interface between the two layers and appear at the top of the lower layer. The top of the lower layer is then made denser by virtue of the particles it now contains and "turns over", moving as a body to the bottom of the tube. This effect is most easily observed in a two-layered system in which a dilute suspension of erythrocytes is used in the upper layer. As shown in Figs. 15 and 16 when the erythrocytes have been centrifuged long enough to move the last of them a few millimeters, many are already at the bottom. Similar effects are observed with multilayered or discontinuous gradients. The results of Kuff and Schneider [11], which yield a picture of layers with material accumulated above each interface, are also due to the turnover effect. It is therefore not surprising that these authors found little microscopically observable difference between the various levels.

Swirling.—It is evident that the acceleration and deceleration of fairly large volumes of fluid (20–100 ml) without mechanical disturbance is very unlikely. The transition from a vertical tube position rotating around an external axis parallel to the tube, to a horizontal position characteristic of useful rotational speeds involves forces which, in many centrifuges, produce a rotation of fluid at the top and bottom of the tubes opposite in direction. The result is often sufficient to produce considerable swirling. A similar condition exists during deceleration.

A somewhat less obvious cause of swirling occurs when a centrifuge is switched off. As shown in one curve in Fig. 3, the maximum rate of deceleration is reached almost instantaneously. Stroboscopic observations with simple dyed layer systems shows that considerable mixing occurs. In non-layered systems the presence of mechanical disturbances is readily demon-

strated by attempting to decelerate centrifuge tubes containing very dilute suspensions of red blood cells in 100-ml centrifuge tubes. Although stroboscopic observation during sedimentation showed a fairly sharp trailing boundary, it was rarely possible to bring the tubes to rest while maintaining the boundary in a completely undisturbed condition.

Convection. –No attempt has been made to study thermal convection currents other than to attempt to minimize them by working from 0–4° C. It should be pointed out that the air resistance in a swinging bucket head such as the one employed here is large and that a considerable amount of power is dissipated. This, together with the discontinuous operation of the cooling system makes it highly unlikely that the temperature in the tubes is either constant or uniform throughout the tube length. The effect of small variations in temperature is greatly magnified as the gravitational field is increased since no increase in the forces opposing movement of the fluid (viscosity) occurs. In a field of 1000 *g*, for example, a change from 0.0 to 0.1° C will effectively change the density of water by 6 mg/ml.

Wall effects.—The major wall effects occur because particles in a centrifuge do not sediment in a parallel fashion, but rather fan out from the axis of rotation. In layered systems, especially those involving thin brei layers close to the axis of rotation and fairly long tubes, a considerable portion of the particles hit the side of the tube before reaching the bottom. Collision with the wall tends to make particles agglutinate and either adhere or slide as a mass to the bottom. The use of sector-shaped tubes to eliminate this difficulty has long been standard practice in the ultracentrifuge. These tubes have not previously been used in the low-speed centrifuge owing, probably, to nonavailability of glass tubes of a satisfactory design.

Aerodynamic effects.—In all instances the centrifuge tubes have been kept closed at the top with either Parafilm[1] or plastic tops.

HIGH RESOLUTION GRADIENT DIFFERENTIAL CENTRIFUGATION

A separation of brei components approaching the ideal has been achieved by use of (a) a continuous-density gradient to minimize hydrodynamic effects and (b) sector-shaped centrifuge tubes to eliminate wall effects, (c) by carefully controlling the centrifuge operation to prevent mechanical disturbances, and (d) by keeping the particles suspended at all times.

Centrifuge operation.—It is evident from the foregoing that considerable care is required during acceleration and deceleration. The most critical

[1] Parafilm, grade "M". Marathon Corp., Menasha, Wisconsin.

period is during the transition of tube orientation from vertical to horizontal. To start the centrifuge the variable transformer (variac) is set to supply enough current to allow the centrifuge to reach a final speed of 210 rpm. At this setting rotation usually begins very slowly but may require a very gentle start by hand. When a speed of 100 rpm is reached, as shown by the stroboscopic control disc, the variac is advanced one division and left there for 10 seconds. The variac is then advanced one division every 5 seconds until a setting is reached which is two divisions above the equilibrium setting for the speed desired. When the desired speed is reached, the variac is turned back to maintain it. Deceleration is accomplished by turning the variac back one setting each 5 seconds until a setting 1 division below the starting setting is reached. This provides a bias current which is sufficient to give a very slow terminal deceleration. Acceleration and deceleration curves for a number of operating speeds are shown in Figs. 2 and 3. For comparison, the deceleration curve observed when the centrifuge is switched off at 3000 rpm is included. The value, $(RPM)^2 \times 1.118 \times 10^{-5}$, which gives the radial centrifugal force when multiplied by the radius, is plotted against time. By integrating the areas beneath the curves it is possible to obtain the number of seconds of operating speed centrifugation equal to that done during acceleration and deceleration. Plots of these are shown in Fig. 4. When the centrifuge compressor is not operated off the same line as the centrifuge motor[1] and a constant voltage transformer is used[2], the values obtained vary by no more than ± 4 per cent. Since acceleration and deceleration generally constitute a minor part of the centrifuge run, a small change in the curves will produce negligible effects.

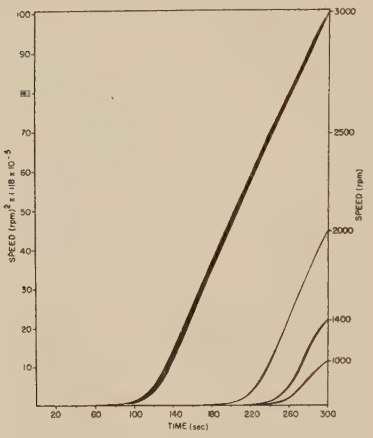
Production of continuous gradients.—The centrifuge artefacts discussed under *hydrodynamic effects* are minimized by dilute breis and continuous density gradients with sugars or other nonelectrolytes, or proteins. In the experiments described here sucrose has been employed.

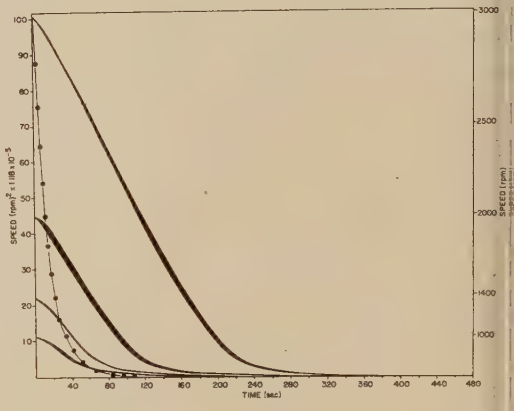
Several methods for producing gradients have been explored. These include modifications of those described by Behrens [1] and the adaptations currently employed in gradient elution procedures [2]. These have not as yet been found as satisfactory as a mechanical system employing differentially driven glass syringes (Fig. 5). This gradient engine produces a slightly sigmoidal concentration versus delivery curve which is of advantage in

[1] Instructions for placing the centrifuge motor and the compressor on separate circuits are available from the manufacturer.

[2] Sola Constant Voltage Transformer, Catalogue No. 30809, Secondary 8.7 amps, 115 volts. Sola Electric Co., Chicago.

separating particles of the size distribution found in liver breis. The solutions delivered by the syringes flow together at a T of 18-gauge stainless steel tubing and then into a small chamber of 1.2-ml volume containing a magnetic mixer. The gradient is delivered to the bottom of a sector centrifuge tube





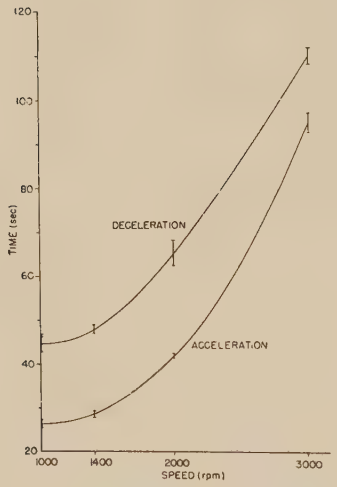


Fig. 2 (upper left). Curves showing controlled accelera- tions. The value RPM² × 1.118 × 10⁻⁵ which is propor- tional to the gravitational force is plotted against time. The area under each curve is proportional to the total force applied during acceleration. The curves show the maximum and minimum values observed at each speed.
Fig. 3 (right). Curves showing controlled deceleration plotted as in Fig. 2. The thin-line curve shows free deceleration as it occurs when centrifuge is suddenly switched off.
Fig. 4 (lower left). Centrifugation during controlled acceleration and deceleration. The results are expressed as equivalent seconds at running speed.

by an 18-gauge stainless steel tube, the lighter solution being delivered first. Each gradient requires approximately $1\frac{1}{2}$ hours to produce. The details of the gradient engine will be described elsewhere.

A very dense solution (generally 80 per cent sucrose) is passed through the mixing system after the gradient is formed to fill the hemisphere formed by the bottom of the sector tube. This layer serves as a cushion to prevent

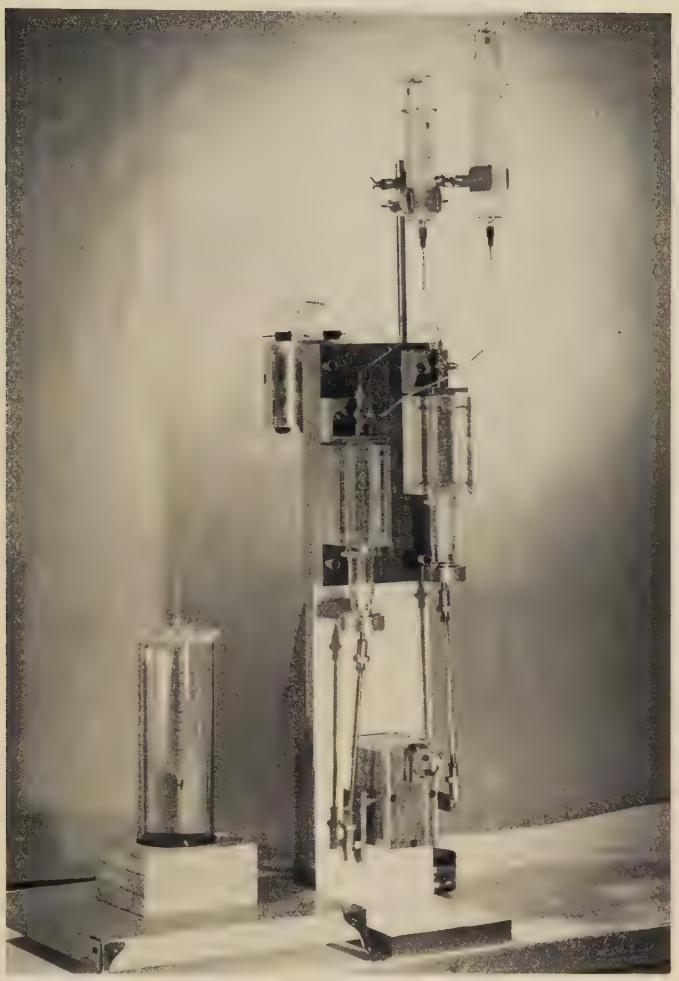
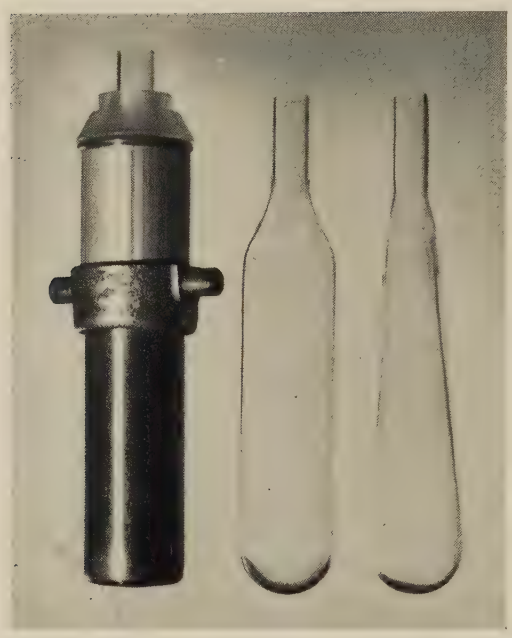


Fig. 5. Model III gradient engine. Two differentially driven pistons continuously mix two solutions of different density to produce a continuous controlled gradient.

large particles from reaching the bottom of the tube. The tubes are kept in ice water during filling and then kept cold until used.

Use of sector tubes.—Round-bottomed, sector-shaped tubes were blown in a suitably machined graphite mold. Two designs have been used. Type A (Fig. 6) is constricted at the top into a round tube 1.4 cm in diameter. This serves to center the tube in the plastic cap shown and to allow the various layers to be removed easily after being raised by a dense fluid introduced to the bottom of the tube by a long stainless steel needle. Although this method of recovering the various fractions is satisfactory for many purposes



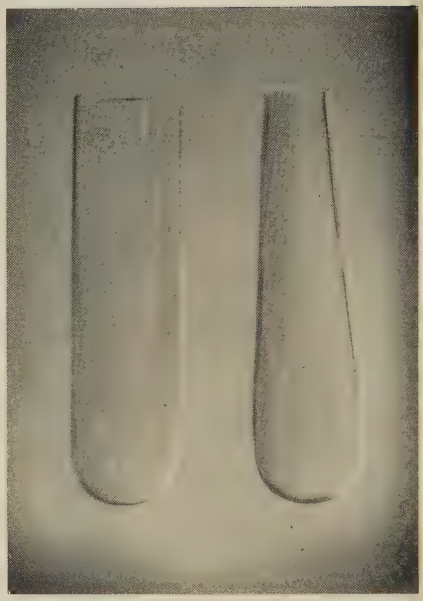


Fig. 6 (left). Type *A* pyrex sector tubes. Two side views illustrate modified sector design. Volume up to constriction is 68 ml. The plastic shield cap shown centers the tube. The aluminum ring decreases the radius sufficiently to allow the tubes to be swung in the Model PR-2 centrifuge. Fig. 7 (right). Type *B* pyrex sector tubes. The flat upper rim allows these tubes to be used with the gradient sectioning device shown in Fig. 8. Useful capacity is 68 ml.

it lacks precision commensurate with the resolution of particles obtained. Unfortunately, the design of the upper portion of the tube is such that the tube will not stand high centrifugal forces. When used in No. 340 shields and No. 325 trunions in head No. 253 with the 3.9-cm spacer sleeves shown in Fig. 6 to decrease the radius of the tubes, half of the tubes shattered at 2400 rpm. Their use is therefore restricted to lower speeds. They have been found especially useful for the isolation of nuclei.

Type B tubes (Fig. 7) allow full advantage to be taken of the inherent strength of the sector design. The upper rim of the tube is ground flat and then fire-polished carefully to remove any tiny cracks. A sliding section device is used to recover samples from Type B tubes (Fig. 8). A stainless steel tube passes through the lower lucite block, bends at right angles in an eliptical hole matching that of the sector tube, and passes to the bottom of the tube. As a dense liquid (80 per cent sucrose, sucrose with $CaBr_2$, or a dense fluorocarbon) is passed to the bottom of the sector tube, the gradient

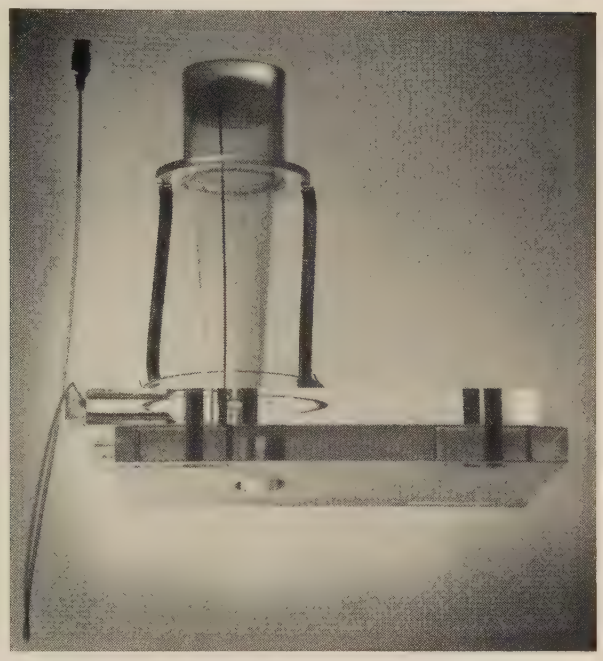
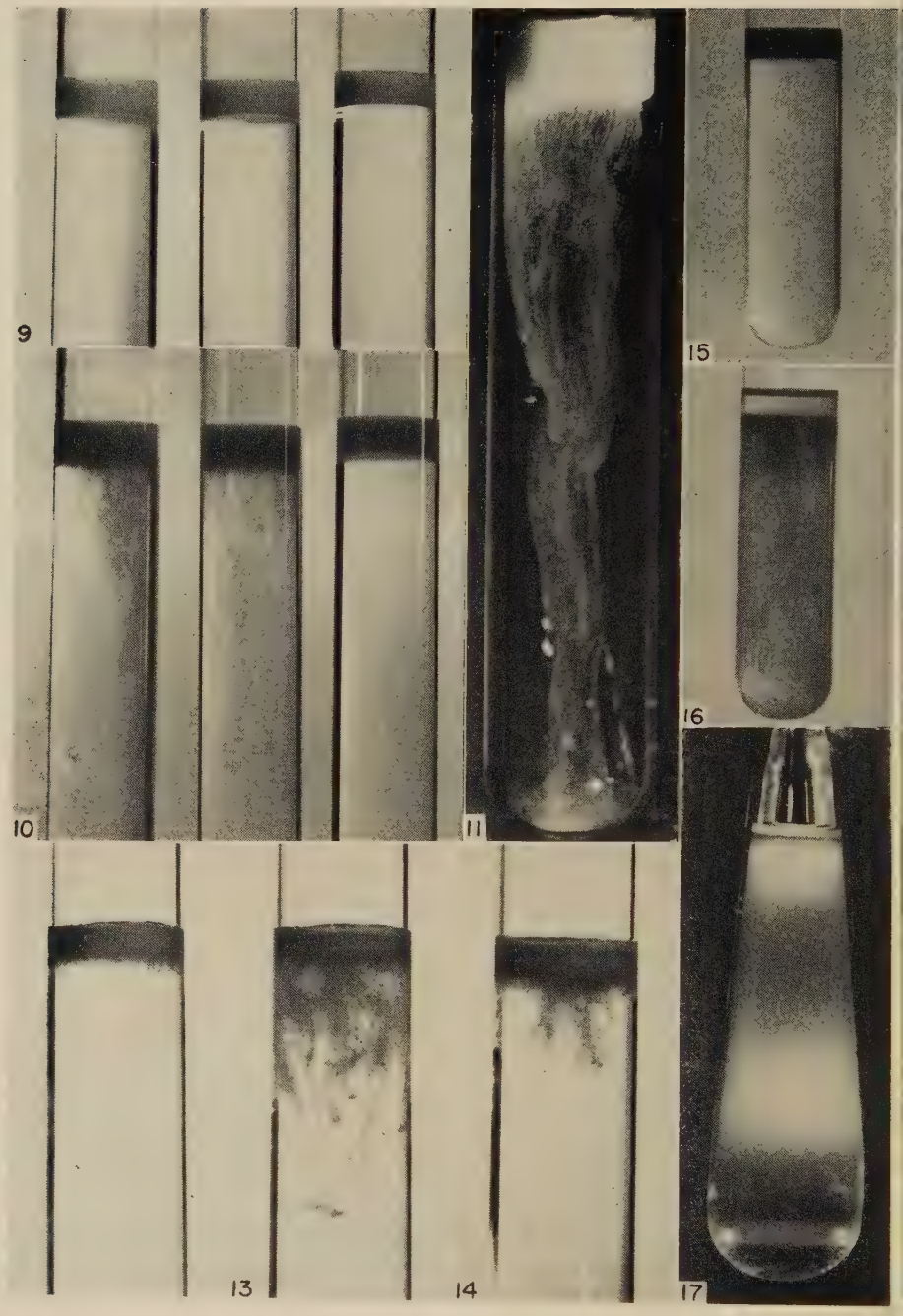


Fig. 8. Gradient sectioning device for use with Type *B* tubes. The gradient is raised by introducing a very dense solution to the bottom of the tube through the stainless steel needle shown.

is slowly raised. Samples of any desired volume up to 5 ml are obtained by sliding the upper lucite block across the lower one. The blocks are aligned by guide pins and are polished to allow visual inspection of the gradients. The entire arrangement fits on an ice bath in such a way that the sector tube is kept cold.

DISCUSSION

The technique described allows a brei to be fractionated into several discrete fractions (nuclei and whole cells, mitochondria, microsomes, supernatant or soluble phase, and lipids) in one step with the quantitative recovery of each fraction (Fig. 17). Data on the sedimentation rates of the various fractions together with photomicrographs will be presented in subsequent papers, as will also a revised method for nuclear isolation. No cross-contamination is observed except for a few particles in the soluble fraction which appear to be mitochondria with an associated lipid droplet. The method is applicable to a wide variety of problems and has already been applied to the isolation of blood platelets and the separation of *Neurospora* conidia according to size. It is now possible to consider studies concerned with quan-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titative changes in the relative proportions of the various fractions. The errors introduced by the presence of whole cells can be corrected at least partially by actual cell counts.

The anomalies occurring in an angle-head centrifuge and their prevention by sucrose gradients have been described by Pickels [12], and the low resolution of the horizontal swinging-tube centrifuge in the absence of a stabilizing gradient has been demonstrated by Kahler and Lloyd [10] who found that glycerol gradients would stabilize the system sufficiently to allow accurate sedimentation rates of polystyrene latex spheres to be obtained. Brakke [3, 4] adapted sucrose density gradients to the isolation of viruses, using a system in which the material to be fractionated was layered on top. He was well aware of anomalous behavior which occurs without a gradient. The gradients were produced by diffusion in a layered system. The author attempted the fractionation of liver breis on soluble starch gradients in 1949 (unpublished).

It should be noted that isopycnic gradient centrifugation for the stratification and separation of marine egg halves and quarters was first described by the Harveys [6, 7]. Gradients of nonaqueous solvents were extensively used by Behrens for the isolation nuclei [1]. Later, Thomson and Mikuta [14] utilized a discontinuous gradient in the separation of rat liver cytoplasmic fractions. Kuff and Schneider [11] have advocated the adaptation of the Brakke technique to the separation of cytoplasmic fractions. Unfortunately in adapting the method to mitochondria and Golgi bodies [13] discontinuous gradients were used and, "the interfaces were carefully maintained". The turnover effect accounts for most of the sedimentation under such conditions and for the accumulation of material above each interface. It is not clear whether the separation of Golgi bodies is due to differential or to isopycnic centrifugation.

Fig. 9. Ten per cent rat liver brei in Solution I layered over (left to right) Solutions II, III, and 0.88 *M* sucrose, containing the same salts used in the isolation medium.
Fig. 10. Same tubes 8 minutes later. No centrifuging.
Fig. 11. Twenty per cent rat liver brei 5 minutes after being layered over Solution III.
Fig. 12. Citrated rat blood 28 seconds after being layered over Solution II.
Fig. 13. Same as Fig. 12 72.5 seconds after layering.
Fig. 14 Citrated rat blood 4 minutes after being layered over 0.88 *M* sucrose.
Fig. 15. Citrated rat blood in 0.25 *M* sucrose (1:4) layered over 30 per cent sucrose.
Fig. 16. Same as Fig. 15 after 5 minutes centrifugation at 100 *g*.
Fig. 17. Separation achieved in continuous sucrose gradient. Nuclei and whole cells form band near bottom of tube, while mitochondria and microsomes are clearly separated above this. Note clear soluble layer above microsomes.

The results presented here suggest that the sedimentation observed in a horizontal tube centrifuge as ordinarily used is not a simple function of the sedimentation rates of individual particles. It does not appear profitable therefore to attempt to predict from Stokes' law what particles would sediment ideally [5] or to infer from apparent sedimentation rates the size of particles whose density is not accurately known [14].

The terminology applied to medium- and low-speed centrifugation is ambiguous and deserves comment. Particles may be isolated by sedimenting them to the bottom of a tube, by sedimenting them part way down through a stabilizing gradient, or by centrifuging them to levels of similar density in a density gradient. The methods in which particles are consecutively sedimented at increasing speeds or times has been termed differential centrifugation. In methods involving gradients, however, the term *density gradient centrifugation* has been used for two very different systems without distinguishing between the use of gradients for stabilization and the separation of particles by allowing them to reach density equilibrium [9]. The following classifications are therefore proposed:

(1) *Differential centrifugation.*—Fractions are collected consecutively at the bottom of the tube. The separation achieved depends on differences in sedimentation rate.

(2) *Gradient differential centrifugation.*—Particles are separated according to their sedimentation rate by centrifuging them through a density gradient which prevents convection currents and other anomalous effects. Fractions may be collected at the bottom, top, or along the gradient. For high resolution, the top layer would be made to approach the theoretically desirable condition of infinite thinness with infinite dilution.

(3) *Isopycnic centrifugation.*—Separation is achieved by suspending the particles in a solution having the same density as the fraction desired. Fractionation therefore depends on particle density. Centrifugation is continued until denser or lighter particles have been sedimented to the bottom or floated to the top.

(4) *Isopycnic gradient centrifugation.*—Particles are separated by sedimenting them through a density gradient until each species finds its isopycnic level and is recovered at that level. Centrifugation is continued until this equilibrium condition is established.

The term *equilibrium centrifugation* is not used for the last method since it has been previously applied to the method in which an equilibrium between sedimentation and diffusion is reached in the ultracentrifuge.

SUMMARY

A number of artefacts which prevent ideal sedimentation have been found during the centrifugation of rat liver breis. These include hydrodynamic effects which cause local areas containing a number of large particles to move as a unit dragging along associated smaller particles, turnover effects at density boundaries, convection currents due to heat and mechanical agitation, and wall effects. Both breis and suspensions of red cells have been used in these studies.

A separation approaching the ideal is obtained by the use of more dilute breis, careful layering over a continuous density gradient sufficiently steep to prevent the anomalies mentioned, and a rigid adherence to a gradual acceleration and deceleration schedule. Gradients of known characteristics are produced by a mechanical gradient engine.

A stroboscopic illumination system has been devised for studying the behavior of layered systems in a refrigerated centrifuge having an observation port. A speed-versus-time recording system allows integration of the centrifuging done. Speeds are accurately measured to 0.02 per cent.

New sector-shaped centrifuge tubes which minimize wall effects and a sliding device for recovering all levels of the gradient from the tube after centrifugation are described. Breis may now be quantitatively fractionated in one or two steps.

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AUTORADIOGRAPHIC STUDY OF INCORPORATION OF P^{32} INTO RIBONUCLEIC ACID AT THE INTRACELLULAR LEVEL

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By the use of autoradiographs measurements of relative amounts of a radioisotope in various parts of a single large cell can be made. For example, Ficq [6] measured the relative rates of incorporation of C^{14}-glycine into RNA and proteins in different parts of the oöcytes of *Asterias rubens*. Taylor [18] compared the relative rates of incorporation of phosphorus-32 into RNA of various parts of the cells of the larval salivary glands and gastric caecae of *Drosophila*. Both studies showed a higher rate of uptake of the isotopes in the nucleolus than in the cytoplasm. In addition, those chromosomes lying adjacent to the nucleolus in the gland cells of *Drosophila* exhibit a rapid incorporation with certain regions being labeled more rapidly than the nucleolus. These observations suggested that this system would yield data to further test the hypothesis that cytoplasmic RNA is derived from that synthesized and secreted by the nucleus [11, 8, 12]. After preliminary experiments to become familiar with the general pattern of incorporation in the third instar larvae of *Drosophila*, data on the relative specific activity-time relations of P^{32}-incorporation in salivary gland cells were collected. These data are presented with some possible interpretations.

MATERIAL AND METHODS

Larvae of *Drosophila repleta*, which are considerably larger than those of *D. melanogaster*, were grown at 23–24°C for 6 to 8 days after egg deposition in the following medium: 15 g agar, 50 g brewer's yeast, and 1 liter of the medium recommended by Atkin *et al.* [1] for yeast, but without the vitamins, and with sugar and minerals trippled in concentration. The pH was adjusted to about 5.0 with propionic acid.

Preliminary experiments to determine the medium most suitable for administering P^{32} indicated that, although the larvae will eat almost any soft food, appreciable incorporation of P^{32} occurs only when the food contains yeast. Suspensions of brewer's yeast in a gelatin or agar medium were used with some success in earlier experiments

[1] The investigation has been supported in part by grants from the U.S. Atomic Energy Commission, Contract AT(30-1)-1304 and from the Higgins Fund of Columbia University.

[18], but regular and uninterrupted feeding are promoted when the labeled food is similar to the one in which the larvae have been growing.

The P^{32}-labeled food used in the experiments reported here was prepared by heating nearly to the boiling point for a few minutes the following mixture in a closed container: 3 ml of the yeast medium of Atkin *et al.* [1], without phosphate, to which had been added 450 μc of P^{32} (specific activity: one mc/0.25 mg of P); enough carmine to color the food; 600 mg of low phosphate yeast, and 25 mg of agar. The pH was adjusted to 5–5.5.

Low phosphate yeast was used in the labeled food in order to maintain the specific activity as high as possible. This yeast was obtained by growing Fleischman's yeast for 48 hours in the medium of Atkin *et al.* [1], with phosphate reduced to 0.1 g of KH_2PO_4 per liter of medium. The cells were washed twice in phosphate-free mineral solution, immediately heated to 60 65°C to kill the cells and inactivate enzymes, and then dried and stored at −10°C until used.

In the first experiments reported here, the qualitative aspects of incorporation were followed in the various cell structures and in the various stages of larval development during the third instar. About one hundred larvae were placed in each vial with labeled food, and transferred to non-labeled food after one or two hours. Larvae were fixed at intervals of one-half or one hour over a period of several hours and the autoradiographs were studied by subjective estimation of grain densities.

In the last experiment reported, grain counts were made above nucleoli and cytoplasm. For this series, about a hundred larvae were placed in a vial of labeled food for two hours, and then transferred to another vial of similar food without P^{32} for three hours. Larvae were fixed at one-hour intervals.

In all experiments larvae were rinsed twice in distilled water before being placed on the labeled food. The rate of eating throughout the experiments was followed by using colored foods (black with India ink, red with carmine, almost colorless when plain) and transferring larvae to foods of different colors at intervals. The food in the gut is completely replaced in 30 minutes to one hour when the larvae are eating vigorously. Only larvae which were feeding regularly were fixed.

At each interval about ten larvae were placed in Carnoy's acetic-alcohol (3:1). After fixation they were embedded in paraffin, cross-sectioned at 7μ, and mounted on slides. Alternate short segments of the ribbon were mounted on each of two slides so that both slides would contain sections of closely adjacent cells from each salivary gland. Tissue sections were passed through xylene to extract paraffin, treated with boiling ether-alcohol (1:3) to extract lipids, and passed through a graded series of alcohols to water. The slides were then treated as follows:

(1) Both slides were treated with 5 per cent trichloracetic acid (TCA) at 2–4°C for 5 minutes. (2) One slide was washed in water and transferred to 70 per cent alcohol until film could be applied. (3) The other slide was digested in a 0.02 per cent solution of ribonuclease (Worthington Biochemical Co.) in distilled water at pH 6.0-6.5 for two hours at 37°C (9), placed in cold TCA for three minutes, rinsed and stored in 70 per cent alcohol until the film was applied.

Autoradiographic stripping film (Kodak, Ltd.) was applied in the manner described previously [7]. After 17 days the autoradiographs were developed in D-19 for 6 minutes at 18°C, rinsed in water and fixed for 10 minutes in Kodak acid fixer. The slides were then thoroughly washed and treated with a hypo eliminator. The prepara-

tions were mounted in Shillaber's refractive index oil ($N_D = 1.46$) for observation with the phase microscope. Bright field was used for examining the silver grains in the emulsion above the cells.

Grain counts were made visually through the entire depth of the emulsion (about 4μ after development and dehydration). Grains were counted above nucleoli and cytoplasm in a circular area of $40\mu^2$, which was delimited by a reticle in the ocular.

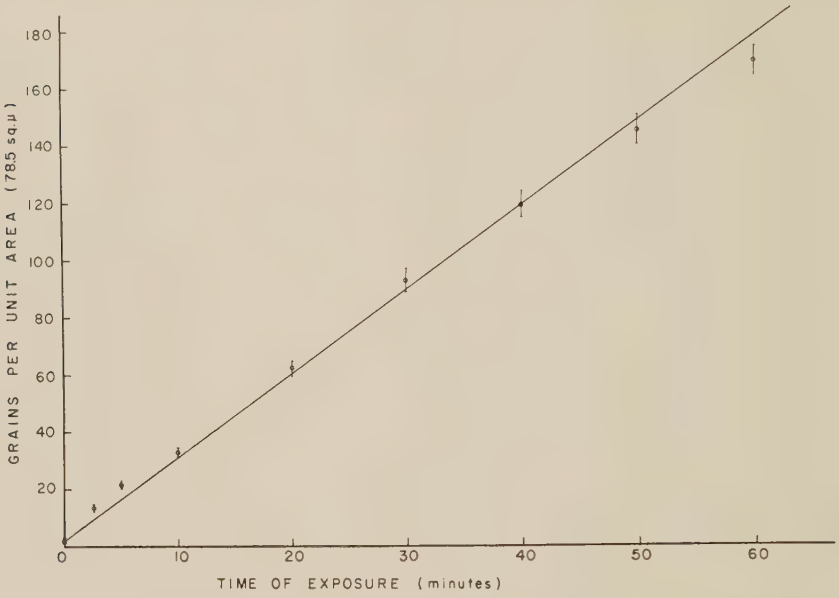

Fig. 1. Graph showing the response of stripping film to β-particles from P^{32}.

To determine whether the response of the film to β-particles was linear in the range of exposures used in these autoradiographs, slides coated with stripping film were exposed to a source of P^{32} β-particles for intervals of 2.5, 5, 10, 20, 30, 40, 50 and 60 minutes. Three slides were exposed for each time interval, except the last two, for which only two slides were prepared. The P^{32} source was prepared by placing a filter paper disk, evenly impregnated with P^{32}-inorganic phosphate, in the bottom of a flat-bottomed dish, the sides of which supported the slide about one inch from the source. After development of the film, the grains were counted in 10 circular areas (of $78.5\mu^2$ each) selected at random near the central portion of each slide. Three slides not exposed to the radiation, but coated and developed at the same time, were used to estimate the background fog of the emulsion. The resulting data are shown in Fig. 1. A regression line was fitted to the points up to 50 minutes by the least squares method using the background value as the y intercept. Corrections were made for decay of the P^{32} during exposure. The vertical bars represent 95 per cent confidence intervals.

Since the response of the film is nearly linear up to about 200 grains per $100\mu^2$, the number of grains per unit area is a valid measure of the relative amount of radiation incident upon the emulsion in this range of exposure.

Although the number of grains is a good estimate of incident radiation, it may not necessarily reflect the absolute concentration of radioisotope in cellular structures below the film. Factors such as geometric arrangement of cellular structures in relation to the film alter this relationship. The estimation of the relative amounts of isotope in various structures to be compared presents fewer problems, provided the distance between objects and film can be considered constant. When the structures are relatively small and separated from other radioactive objects so that nearly all of the grains produced in the film can be counted, direct comparisons may be made. If structures vary considerably in size and shape and only part of the grains are counted certain correction factors are necessary, some of which have been considered by Nadler [13]. In the data presented here, counts from cytoplasm from different cells may be compared directly without correction. Furthermore, since the areas counted were always over sections of cytoplasm free of nuclear materials, there was no variation from that source. On the other hand, all sections contain cytoplasm which may interfere with counts over nucleus or nucleolus. Since the nucleolus usually lies near the center of the nucleus, proper selection provided nucleoli with negligible interference from the cytoplasm. Interference from chromatin was unavoidable; therefore, the counts over nucleoli reflect relative changes in isotope concentrations in cylindrical plugs of nuclei which always included a nucleolus. Since the nucleoli varied little in size, comparisons between these counts at different hourly intervals are also valid. However, comparisons of isotopic concentrations of cytoplasm and the cylinders of nuclear material containing the nucleolus would require correction factors which are difficult to calculate precisely since the proportion of P^{32} in chromatin and nucleolus varies, particularly during the first two hours of the experiment.

OBSERVATIONS AND RESULTS

The salivary gland during early third instar is a long, narrow sac, the posterior three-quarters of which is of nearly uniform diameter. In cross-section, 6–7 cells are arranged around a central lumen. The cells have extremely basophilic cytoplasm, nucleoli, and chromosomes. Throughout the third instar the cells increase in size, and basophilia gradually decreases. Soon after mid-third instar a rapid enlargement of the gland begins which soon

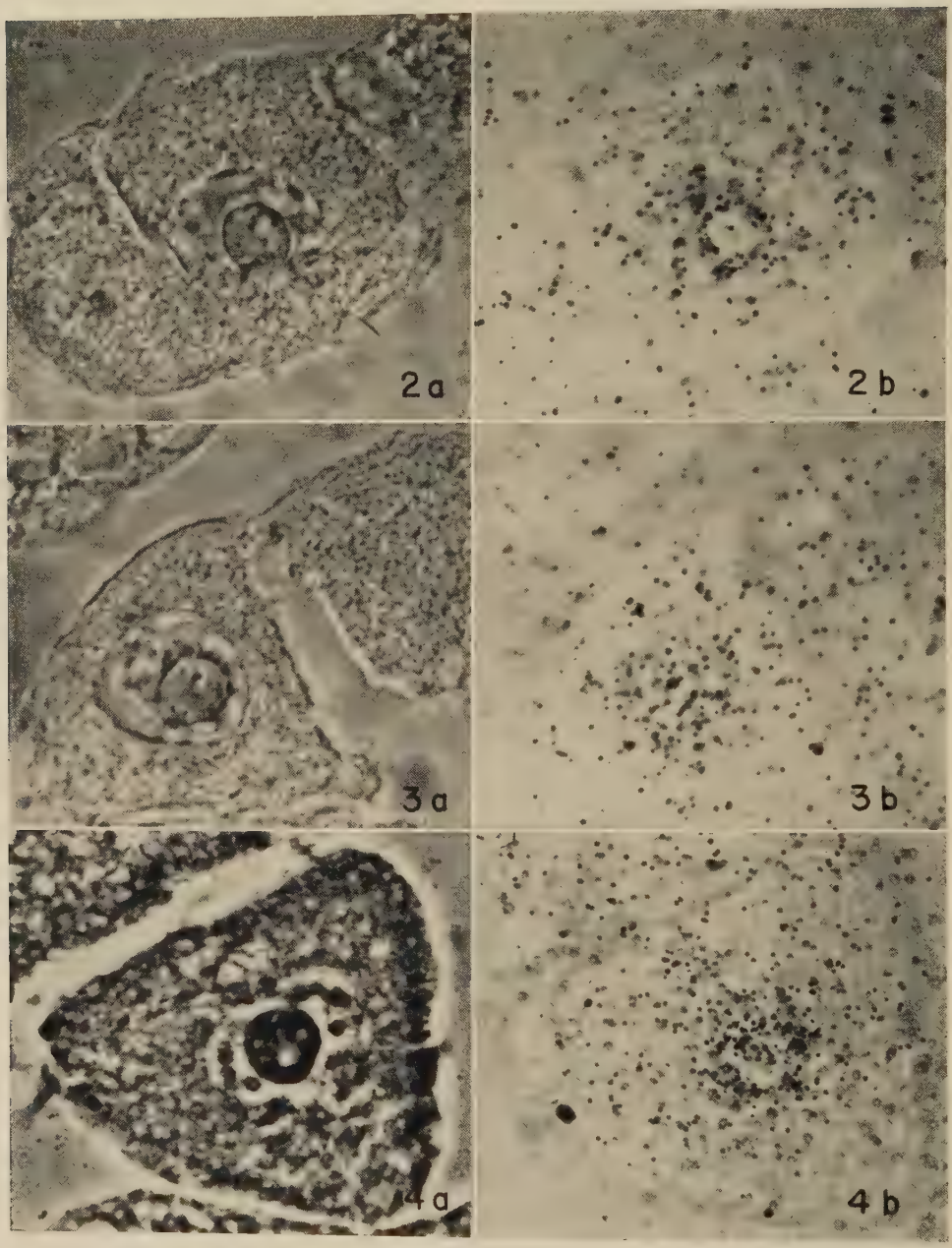

Fig. 2 a. Cell from salivary gland of a larva that had been eating labeled food for 1½ hours.
Fig. 3 a. Cell from salivary gland of a larva 2½ hours after beginning to eat labeled food.
Fig. 4 a. Cell from the gastric caeca of a larva fed 1½ hours on food containing P^{32}. Phase photographs; mag. ×1200.
Figs. 2 b, 3 b and 4 b. Bright field photographs at the level of the emulsion over the respective cells.

results in the structure used so often in cytogenetic studies. Polysaccharide granules appear in the cytoplasm, and basophilia is markedly reduced.

The cells used in this study were from mid-third instar larvae, when all the cells of the salivary gland are of fairly uniform appearance and no polysaccharide granules were visible with the phase microscope. Uptake of P^{32} was consistently high in all glands and all cells in this stage. In the last third of this instar, P^{32} uptake decreases to such an extent that none is detectable by autoradiographs. At this time obvious changes are occurring in the cells as shown by the large differences in basophilia and polysaccharide content among the cells of a gland. By the end of the instar all cells are weakly basophilic and are filled with the polysaccharide-containing granules.

Autoradiographs prepared from larvae fixed at intervals after they began to eat the labeled food, demonstrate the pattern of intracellular incorporation in mid-third instar larvae. The first structures in which P^{32} can be detected are sectors of chromosomes adjacent to the nucleolus. Peripheral material of the nucleolus is labelled almost simultaneously. (See Figs. 2 a and 2 b.) Most of the cells present this picture about one to one and one-half hours after the larvae are placed on labeled food. In some of the cells the highest concentration of grains appears over the nucleolus, but this may be caused in part by chromatin of high activity lying immediately adjacent to the nucleolus, and sometimes nearly above or below it. Within three hours after feeding, the nucleolus is definitely the site of highest radioactivity in nearly all the cells (Figs. 3 a and 3 b). The cytoplasm by this time also has a readily detectable amount of P^{32}. Within 5 or 6 hours the cells are nearly uniformly labeled in nucleolus and cytoplasm.

The basophilia and pattern of intracellular incorporation in the cells of the gastric caecae are similar to those of the salivary gland during the first half of third instar. Certain cells at the base of the caecae, however, incorporate P^{32} into RNA at a rate exceeding those of the salivary gland. For this reason the differential labeling of chromosomes and nucleolus often shows better than in the salivary glands (Figs. 4 a and 4 b).

Most of the P^{32} remaining after cold TCA treatment is removed by digestion of sections with ribonuclease; this large fraction is therefore assumed to be RNA phosphorus. The residual P^{32}, presumably in a phosphoprotein, is small except during the early stages of the rapid enlargement of the gland cells, when there is no longer any detectable incorporation into the RNA fraction of nucleus or cytoplasm.

The data on grain counts above nucleoli and cytoplasm for a five-hour period after the larvae were placed on labeled food are given in Table I and

TABLE I

Number of silver grains in autoradiographs over nucleoli and cytoplasm (circular area 40 μ^2).

Hours after larvae began eating P^{32}	Number of larvae	Number of cells	Nucleolar diameters (μ) with S.D.	Grains after cold TCA with S.E.	Nucleolar diameters (μ) with S.D.	Grains after RNase with S.E.	Difference with S.E.
	cytoplasm*						
1	3	20		1.4±0.3		0.1±0.2	1.3±0.3
2	4	20		8.4±1.3		1.2±0.5	7.2±1.4
3	3	20		11.5±0.6		2.1±0.4	9.4±0.7
4	4	20		23.1±0.9		2.7±0.3	20.4±1.0
5	2	20		26.9±1.0		2.3±0.5	24.6±1.2
	nucleoli*						
1	3	20	7.3±0.2×7.4±0.4	4.1±0.5	7.2±0.3×7.4±0.4	1.0±0.3	3.1±2.0
2	4	20	7.2±0.2×7.7±0.5	38.7±2.7	7.2±0.2×7.4±0.4	7.8±1.2	30.9±3.0
3	3	20	7.7±0.7×8.2±0.8	47.0±1.3	7.6±0.5×7.8±0.6	5.1±0.6	41.9±1.5
4	4	20	7.8±0.6×8.4±0.9	47.8±1.6	7.1±0.7×7.9±1.0	6.2±0.4	41.6±1.7
5	2	20	7.3±0.4×7.9±0.7	38.1±1.1	7.3±0.8×7.9±0.8	4.1±0.7	34.0±1.4

* Mean values for background fog of emulsion have been subtracted.

Fig. 5. The grain counts show clearly what was observed subjectively in the earlier experiments. Radioactivity of the nucleolus is very high by the end of the second hour, and has reached its maximum value at the end of the third hour. The cytoplasmic activity, however, increases slowly throughout the five-hour period, with what appears to be a leveling off in the fifth hour. By the end of the fifth hour the radioactivity of nucleolus and cytoplasm are approaching the same level but the nucleolus is still significantly higher.

DISCUSSION

Since little is known of the function of the salivary glands of *Drosophila*, other than that they probably secrete substances concerned with digestion, it is rather fruitless to attempt a discussion of the data presented here in reference to the role of RNA in the salivary gland cell. The observed incorporation of P^{32} into RNA of chromosomes, nucleoli, and cytoplasm must indicate that these structures participate in RNA synthesis. But whether the sites of incorporation are also sites of RNA function is unknown. Conversely, the absence of P^{32} uptake into RNA in late third instar cannot be taken as evidence that the functioning of RNA has ceased in these stages, although

it indicates that synthesis is not occurring. The data are most applicable to a consideration of the RNA metabolism of the various cellular structures, regardless of the function of RNA.

Many chemical studies have reported the occurrence of a rapid incorporation of P^{32} into the RNA of the nucleus compared to that of the cytoplasm. The usual chemical techniques have not distinguished, however, between

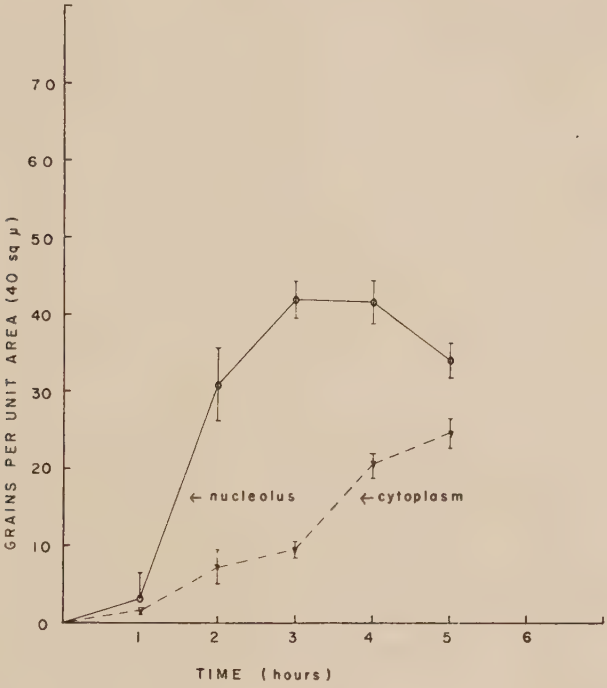

Fig. 5. The change in the number of grains in autoradiographs over cytoplasm and nucleoli of cells of the salivary glands at various times after the larvae began to eat labeled food.

RNA of the chromatin and that of the nucleolus. The autoradiographic studies have demonstrated that both of these nuclear fractions have a more rapid rate of incorporation than the cytoplasm. It is tempting to conclude either (*a*) that the nuclear RNA is a precursor of the cytoplasmic RNA, or (*b*) that the nucleus is very active in RNA metabolism while the cytoplasm is not—assuming that nucleus and cytoplasm have a common precursor. A recent mathematical analysis of the course of P^{32} incorporation into the various cellular RNA fractions [3, 4] has disagreed with both these hypotheses. The analysis indicated that nuclear and supernatant cytoplasmic RNA have a common precursor and that supernatant RNA is the precursor of the RNA of the cytoplasmic particulate fraction. The turnover rate for

nuclear RNA was calculated to be similar to that of most of the other RNA fractions, when the precursor activity was considered.

A similar analysis of the possibility that nucleolar RNA is the precursor of cytoplasmic RNA (which should include both the supernatant and particulate fractions) can be made with the data presented here if certain assumptions are made. Since the basophilia of cytoplasm and nucleolus are similar and change very slowly during the first half of third instar, the RNA concentrations in nucleolus and cytoplasm will be assumed to be equal and to remain constant during the five hour period of the experiment. Measurements of nucleoli, nuclei and total cell size indicated that the volumes had a ratio of about 1:10:100, which changed little during the course of the experiment (see measurements in Table I). It is assumed then that the cytoplasm contains 100 times as much RNA as the nucleolus.

The equation of Zilversmit *et al.* [19] as formulated by Barnum and Huseby [3] relating precursor and product is as follows:

$$\frac{dA}{dt} = \frac{v}{a}(I - A). \tag{1}$$

For the special case presented here, I and A are the relative specific activities of precursor and product, respectively (grains produced per unit volume if concentrations are assumed to be equal); v is the rate of conversion of precursor (i), in which the unit is the amount per nucleolus, to the product (a). The dimensions of v are the number of nucleolar units of RNA per hour per cell; a is the number of nucleolar units of RNA present in the cytoplasm of the cell; and dA/dt is the rate of change of the relative specific activity of product, a. The above equation was derived on the basis of the following assumptions: (i) a steady state, i.e., a and i are constant over the interval during which the calculation is made; (ii) the rate of appearance and disappearance of the compound must be constant during the time interval used for calculation; and (iii) the appearance and disappearance of all molecules must proceed at random, i.e., the organism does not distinguish between "old" and "newly formed" molecules.

When equation (1) is written in the integrated form:

$$A = \frac{v}{a}(\iota - \alpha) \tag{2}$$

and the areas under the specific activity-time curves for nucleolus and cytoplasm (Fig. 5) are substituted for ι and α, respectively, a turnover rate

for nucleolar RNA may be estimated. (A represents the total change in the cytoplasmic activity.) The value of v is found to be 29.3, i.e., the fractional turnover per hour for the nucleolar RNA if it were contributing the entire activity found in the cytoplasm. Now if a smooth specific activity-time curve for the cytoplasmic RNA is drawn as near the experimental values as possible (curve B in Fig. 6) and the curve for a theoretical precursor is cal-

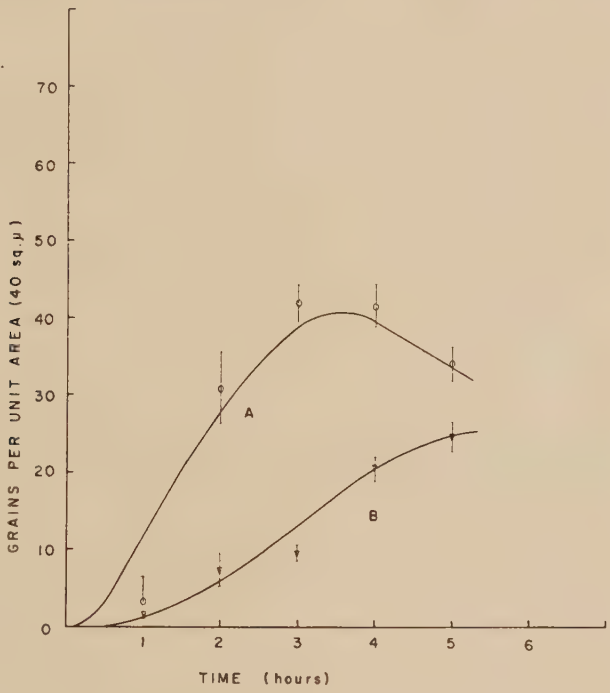

Fig. 6. A smooth curve (B) drawn to show the change in relative specific activity of cytoplasmic RNA and its theoretical precursor (curve A); the grain counts of nucleolar RNA also are plotted to show the correlation between theoretical and experimental values.

culated by means of formula 1 with v equal to 29.3, the curve A (Fig. 6) is obtained. It may be noted that the experimental values for the nucleolus fall close to this curve, and therefore, the nucleolar RNA fits the role of a precursor for cytoplasmic RNA in these cells.

To facilitate comparison with the alternate hypothesis, i.e., that the nucleolar RNA and the cytoplasmic RNA have a common precursor, the data are plotted in another way in Fig. 7. First, equation 2 is written in the form:

$$C = \frac{v}{a}(\nu - \gamma) \tag{3}$$

where C is grains per unit area over cytoplasm and ν and γ are the areas under the activity-time curves for nucleolus and cytoplasm, respectively. C is plotted

against $(\nu-\gamma)$ in Fig. 7 a. The values for γ and ν were determined graphically from the smooth curve drawn for cytoplasmic points in Fig. 6 and from a smooth curve drawn through all of the nucleolar points. It may be seen from equation (3) that a straight line passing through the origin should result if the data fit the hypothesis that the labeled nucleolar RNA is the sole precursor of the labeled cytoplasmic RNA.

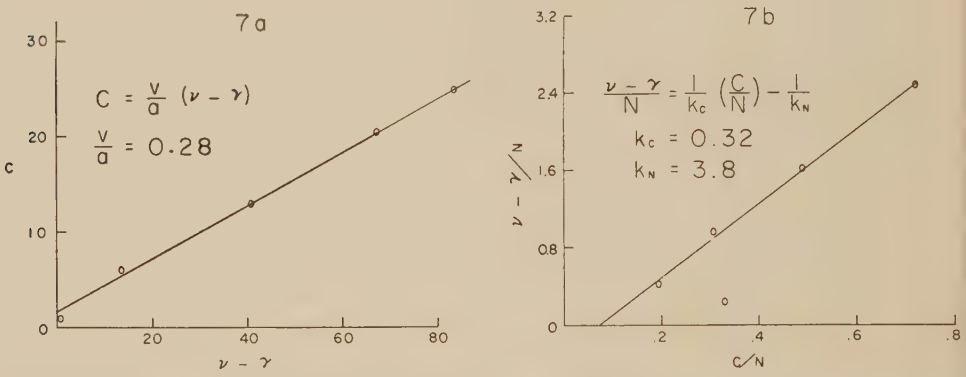

Fig. 7. The graphical solutions of equations (3) and (6) to test the hypothesis that the nucleolar RNA fits the role of precursor of the cytoplasmic RNA (7 a) and the alternate hypothesis that the two types of RNA have a common precursor (7 b).

Now the data are plotted in Fig. 7 b to test the hypothesis that nucleolar and cytoplasmic RNA have a common precursor. From the general equation (2), the following equations may be written:

$$C = k_C(\iota - \gamma) \tag{4}$$

$$N = k_N(\iota - \nu) \tag{5}$$

where for each time interval C and N are the total change in cytoplasmic and nucleolar activities, respectively, ι is the integral of the specific activity of the hypothetical common precursor and γ and ν are the integrals of the relative specific activities for cytoplasm and nucleolus, respectively. Each equation is solved for ι and the two identities are set equal to each other. Dividing through by N and transposing, the following equation is obtained:

$$\frac{\nu-\gamma}{N} = \frac{1}{k_C}\left(\frac{C}{N}\right) - \frac{1}{k_N}. \tag{6}$$

According to this equation, when $\frac{\nu - \gamma}{N}$ is plotted against C/N the points should fall on a straight line if the data fit the hypothesis. With the exception of the first hour, the points fit a line the slope and intercept of which indicate that the nucleolar RNA turns over about 4 times an hour, while the turnover for cytoplasmic RNA is 0.3 per hour. These values, as in the first calculation are based on the assumption that the RNA concentration is the same in nucleolus and cytoplasm.

It is not possible, of course, that the nucleolar RNA is the sole source of cytoplasmic RNA, according to the first hypothesis, and that the alternate hypothesis, that nucleolus and cytoplasm have a common precursor, is also true. However, if the RNA of the nucleolus turned over fast enough to remain almost in equilibrium with its precursor, which was also the precursor of cytoplasmic RNA, the distinction between the two hypotheses would not become apparent with these data. The hypothetical common precursor could be produced by the chromosomes as suggested by the early appearance of labeled RNA in certain chromosome sectors near the nucleolus, but more data are necessary for any definite conclusions on this point.

These calculations cannot definitely answer the question of whether there would be any turnover in the cytoplasmic RNA independent of the nucleus. Some data on this point has been obtained for C^{14}-labeled compounds. Enucleated sea urchin eggs incorporate some C^{14}-glycine in RNA [10]. Szafarz and Brachet [5, 17] likewise demonstrated that RNA of enucleated fragments of the alga, *Acetabularia*, incorporated C^{14} from orotic acid, but the amount of the RNA in the enucleated fragments decreased at a much more rapid rate than in nucleated fragments. Enucleated halves of amoebae also were shown to lose RNA at a much faster rate than nucleated halves treated in a similar way [7].

As pointed out by Smellie *et al.* [16] and others, marked differences in molar composition of the RNA's of the nuclear and cytoplasmic fractions appear to preclude as simple a precursor-product relationship as the direct transfer of RNA from nucleus to cytoplasm. The observation that the composition of nuclear RNA varies, however, suggests heterogeneity in this fraction. In addition, the nuclear fractions used for chemical analyses always contain nucleolar and chromatin RNA, which were found in this study to have different patterns of incorporation. In view of the very likely heterogeneity of most isolated nuclear RNA fractions, it seems possible that the metabolic behavior of this fraction may vary according to its composition. In at least one case [14] it has been found that nuclear and particulate RNA's

have the same composition and therefore a direct exchange of RNA between nucleus and cytoplasm is not precluded.

Recent evidence of another type indicates that large molecular materials may be transferred from nucleus to cytoplasm. Pollister *et al.* [15] have demonstrated by means of the electron microscope that in early frog oöcytes, unbranched thread-like structures project through the nuclear membrane and end in the cytoplasm. Near the outer surface of the membrane are numerous particles similar to this material which is also found in the nucleus, sometimes with strands extending into the nucleolus. The nuclear membrane of the cells of larval salivary glands of *Chironomus* is also reported to have a fine structure consisting of pores that would allow the passage of large molecules [2].

SUMMARY

By means of autoradiographs the incorporation of P^{32} into RNA of cytoplasm, chromosomes and nucleoli of larval salivary glands of *Drosophila* was studied. Larvae were fed for one or two hours on food containing P^{32}-inorganic phosphate and then transferred to similar food without the isotope for the remainder of the experiment. Incorporation is uniformly high in all cells of the gland during the first half of the third instar, but falls in the latter half of this stage so that labeling of RNA cannot be detected in the last third of the instar.

The intracellular pattern of incorporation is such that labeled RNA can be detected after the first hour in sectors of chromosomes lying near the nucleolus. Almost simultaneously some P^{32}-labeled RNA appears in the nucleolus. Within three hours the amount of RNA-P^{32} in the nucleolus has increased so that one-half or more of the total in the nucleus is localized in the nucleolus. By this time labeled RNA is readily detectable in the cytoplasm, and within 5 or 6 hours the grain count over cytoplasm is nearly as high as over the nucleolus.

A study of the relative specific activity-time relations of cytoplasmic and nucleolar RNA based on grain counts in autoradiographs shows that nucleolar RNA fits the role of precursor of cytoplasmic RNA. However, the data also fit the hypothesis that nucleolar and cytoplasmic RNA have a common precursor. These data are discussed with reference to the hypothesis that cytoplasmic RNA is synthesized in the nucleus, probably in the chromosomes, and transferred to the cytoplasm, at least in part, by way of the nucleolus.

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DESOXYPENTOSE NUCLEIC ACID SYNTHESIS DURING MICROSPOROGENESIS IN *TRADESCANTIA*[1]

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Recent experimental evidence indicates that of all the constituents of the nucleus so far analyzed, the desoxypentose nucleic acid (DNA) is quantitatively the most consistent and predictable. The constancy of its content in nuclei of various tissues of an organism, and among individuals of a species has been demonstrated in a wide variety of animal and plant cells by both biochemical and cytophotometric techniques and has been closely correlated with chromosome complement (see discussions in [28, 36, and 42]). Such observations suggest a parallelism between DNA and chromosome behavior which could serve as a biochemical basis for understanding hitherto unresolved cytological and genetic questions.

There is still disagreement over the timing and nature of chromosome duplication during the mitotic cycle, a problem that has occupied cytologists since the significance of this precise phenomenon was first understood (see [13], for discussion). Although the measurement of DNA content of single cells by cytophotometric methods [27, 16] has permitted a direct correlation of cytological and biochemical events, disturbing discrepancies in reports of DNA content and behavior during cell division exist which need to be resolved (see [36], for discussion). The information, however, is at least consistent in pointing to the occurrence of DNA synthesis at some time prior to mitotic prophase, probably during a specific period in the interphase [34, 21, 43, 24]. These observations have been borne out by P^{32} experiments using autoradiographic techniques to locate and analyse cells in which P^{32} has been incorporated by the DNA fraction [11, 12, 38, 39, 25].

A unique analytical opportunity is provided by the simultaneous use of the cytophotometric and autoradiographic techniques. By determining DNA

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content together with DNA-P^{32} uptake of single cells, the timing of DNA synthesis may be verified by two independent means and, a point of considerable importance, the relationship between incorporation and synthesis may be examined at the cellular level. This paper presents in detail part of the work following this approach reported earlier in abstract form [18]; the concurrent cytophotometric experiments designed to resolve some of the above-mentioned discrepancies in the literature will be reported elsewhere and only the pertinent measurements included here. Two experiments will be discussed: the first establishes the nature of the fractions incorporating P^{32} and the second correlates cytophotometric and autoradiographic information.

MATERIALS AND METHODS

Anthers of a clone of *Tradescantia paludosa* Anderson and Woodson [1] (Sparrow clone B-2-2, derived from Sax clone 5) were used as a source of material. Inflorescences of *Tradescantia* are double helicoid cymes [2] which may contain flowers ranging in development from early bud primordia to open flowers. Usually one flower opens each morning on alternate sides of the double inflorescence. Occasionally one flower opens on each side or a day passes without a flower opening. However, observations indicated that in those inflorescences with meiotic prophase being initiated in the anthers of the fifteenth or sixteenth bud below the first open flower, opening of one bud per day is fairly regular with the appropriate temperature range. Therefore, the development from the initiation of meiotic prophase to mature pollen requires about 16 days and the buds in such inflorescences differ approximately 24 hours in developmental stage.

Freshly cut inflorescences with stems 3 inches long were placed for 28 hours (25°C) in a mineral solution [37] containing 20 μc of P^{32} per ml with a specific activity of 0.146 mg of phosphorus per millicurie. Since geiger counts showed that significant amounts of P^{32} reach the buds in 3–4 hours, the cells may be assumed to be incorporating labeled phosphorus for a period of 24 hours. One inflorescence then yields cells that have been exposed to P^{32} at all stages in development.

Material was fixed in Carnoy's alcohol-acetic acid (3:1), either as whole anthers for sectioning or as smears. Since the sporogenous cells of a locule are highly synchronized in development successive groups of serial sections were distributed to 5 or 6 slides, which served as replicates for comparison after different treatments. After removal of paraffin from sections with benzene and washing in absolute alcohol, slides were placed for 5 minutes in boiling ether-alcohol (1:3) to remove any phospholipids not removed during fixation. Tissues were hydrated in a graded alcohol series and extracted 5 minutes in 5 per cent trichloracetic acid (TCA) at 2–4°C to remove any remaining soluble nucleotides. After washing in water, one of the replicate slides was stored in cold 50 per cent alcohol. The remaining slides were treated as follows:

(a) Two slides were placed for 10 minutes in *N* HCl contained in a vial in a water bath at 60°C (measurements showed this to be optimal hydrolysis for the Feulgen

reaction). Slides were rinsed in ice water and stained by the Feulgen reaction [7]. One slide was stored in cold 70 per cent alcohol until it could be coated with stripping film, and the other was mounted for cytophotometric measurements. (b) One slide was placed for 2 hours at 37°C in a 0.02 per cent solution of protease-free crystalline ribonuclease (Worthington Biochemical Co.) adjusted to pH 6.0. (c) In some experiments one slide was digested as in (b) above and then transferred for 2 hours at 37°C to a 0.01 per cent solution of desoxyribonuclease (Worthington) adjusted to pH 6.0 in 0.005 *M* $MgSO_4$. (d) When desoxyribonuclease was used another slide was hydrolyzed for 30 minutes in 5 per cent TCA at 90°C for total removal of nucleotides.

After removal from enzyme solutions slides were rinsed for 3 minutes in 5 per cent TCA at 2–4°C to remove any soluble nucleotides. Slides were stored in cold 70 per cent alcohol until stripping film could be applied. Autoradiographic stripping plates (Kodak, Ltd., London), which have 5 microns of emulsion on a backing of 10 microns of gelatin, were used. The film was cut into rectangular pieces while still attached to the glass plate. The pieces were stripped off with cover glass forceps and spread emulsion side down on the surface of water at 25°C. After the film was thoroughly flattened and expanded to an equilibrium condition (2 minutes on the water surface), slides were immersed in the water and raised under the film strips. The preparation was dried and stored in plastic slide boxes for exposure (2 weeks). Development was for 6 min. at 18°C in Kodak D-19. After fixing 10 min. in Kodak acid fixer slides were thoroughly washed, dehydrated in an alcohol series and mounted in balsam or oil (index of refraction 1.47). The oil allows examination of the unstained sections without the phase microscope. Grains were counted by using in the eyepiece a reticle marked with concentric rings. The two inner rings covered an area of 200 sq. μ in the specimen. This covers an area considerably larger than the nucleus (diameters 7–10 μ) and reduces the variations in grain counts due to differences in nuclear size, shape and distance from the emulsion. For a discussion of these factors refer to Gross *et al.* [9] and Nadler [19].

Cytophotometric procedure.—Although, for purposes of comparison, two methods of measuring absorption of the Feulgen complex in nuclei were used, only the data obtained with one will be reported here. This method differs from that of measuring extinctions through a central "plug" generally used [16] and relies on measurements of the whole structure at two carefully selected wavelengths as described by Ornstein [20] and Patau [23]; the computational procedure suggested by the latter author was found to be especially convenient. In order to yield reliable data, the "plug" method must be used with careful regard for the nature of the material and the potential errors, sometimes difficult to minimize experimentally, such as those due to high extinctions, distribution of absorbing material (i.e. DNA-Feulgen complex) unevenly or in clumps and determining the size and shape of nuclei. As is to be expected theoretically and as will be demonstrated elsewhere, the "two-wave length" (2 λ) method has advantages in that it either avoids or makes corrections for these errors. It is consequently more reliable and is the method of choice, particularly where such a wide assortment of conditions involving extremes of size and shape, of distribution and concentration is encountered as in the various stages of *Tradescantia* microsporogenesis.

In the 2 λ method, the cytophotometric apparatus used embodies features described by Pollister [26] and Moses [16]. Energy was isolated by a Bausch and Lomb

250 mm grating monochromator with entrance and exit slits set at 0.3 mm to provide an over-all spectral purity of 4 mμ with about 75 per cent of the energy falling within 1 mμ each side of the selected wave length. The system was set up for critical illumination with a 16 mm (10×), N.A. 0.3 Bausch and Lomb apochromat objective used for sub-stage condenser. The microscope objective was a 3 mm, N.A. 1.40 (61×) Bausch and Lomb apochromat and the ocular a 7.5× coated compens. Flared light and light scattered by internal reflections were estimated and together amounted to less than 2 per cent. Such instrumental calibrations as phototube cathode centering, linearity of response of the phototube-galvanometer system, etc. [16] were routinely employed. The measuring procedure followed the two-wave length (2 λ) method proposed by Ornstein [20] and by Patau [23]; these authors are referred to for a full discussion and defense of the method. In our hands, the procedure was as follows: Initially, absorption spectra were obtained of the Feulgen-nucleal complex in small homogeneous volumes of representative nuclei throughout the pollen maturation cycle. Because of the extremely high extinctions (1.0–3.0) encountered in many cases at the absorption maxima, the two wave lengths to be used in actual measurements were selected in an off-peak region where their extinctions would be in the range .2 to .6. The region 460 to 490 mμ was chosen because of the higher instrumental sensitivity at this end of the spectrum and because the slope of the curve here is less than on the longer wave length side of the peak, a factor which should reduce errors due to spectral impurity. Four wave lengths in this region were chosen so that they fell on a nearly linear portion of the absorption curve, and for each stage to be analyzed, the extinctions through small homogeneous areas of 5 representative nuclei were determined at each wave length. 470 mμ was arbitrarily selected as λ_1 and the wave length giving an extinction twice that at λ_1 was interpolated and taken as λ_2. The remarkable constancy of λ_2 (487 mμ) observed from slide to slide and preparation to preparation indicates the stability of the Feulgen-DNA complex and the small variation in homogeneity of the test areas.

Considerable care was taken to select only whole nuclei for measurement; the diaphragm below the phototube was closed to the smallest area that would contain the entire outline of the nucleus, and the transmission measured at λ_1 and λ_2. Relative amounts of absorbing material were computed according to the convenient method proposed by Patau [23]. No attempt has been made in these experiments to determine k, the specific extinction coefficient for the Feulgen-DNA complex, which would permit expression of the data in terms of absolute rather than relative units. The advantage of thus disclosing a possible constant factor relating an external standard to the cytochemical data is irrelevant to the purpose of these experiments and would hardly outweigh the difficulty of obtaining and interpreting the initial standard for the Feulgen reaction [29, 14]. Hence, it has been assumed that k is constant for identically and simultaneously prepared material within an experiment and comparisons have been made accordingly.

The error of reproducibility with this method was estimated by 20 repeated measurements of the same nucleus and was found to be (SD of the mean) 1.93 per cent. This error includes variations in light source intensity combined with response of the phototube-galvanometer circuit (0.5 per cent), variability in setting the circumscribing diaphragm (1.0 per cent) and variations in setting correct wave lengths with the monochromator (0.5 per cent).

RESULTS

Autoradiography.—In an effort to gain some information on the relative rates of incorporation of P^{32} in the various nucleoprotein fractions and the effectiveness of the procedure to detect DNA-P^{32}, the differences in concentrations of silver grains in autoradiographs over nuclei were determined after the following treatments:

(1) Fixative (acetic-alcohol), lipid solvents and cold TCA.

(2) Fixative, lipid solvents, cold TCA and ribonuclease.

(3) Fixative, lipid solvents, cold TCA, ribonuclease and desoxyribonuclease.

(4) Fixative, lipid solvents and HCl hydrolysis (1 N HCl, 10 min. at 60° C).

(5) Fixative, lipid solvents and hydrolysis in 5 per cent TCA at 90° C for 30 min.

Differences in number of grains counted per 200 square microns, correcting for a background count, may be interpreted in terms of relative concentrations of P^{32} incorporated in nucleoproteins and other phosphoproteins. Figure 1 presents a summary of these data from several inflorescenses. Differences in number of grains between cells on slide (1) with all nucleic acids and phosphoproteins intact and slide (2) with all of the RNase-digestable RNA removed represents a relative measure of RNA-P^{32}. The difference between slide (1) and slide (4) also represents RNA-P^{32} or at least the easily hydrolyzable nucleic acid which may also contain a small amount of DNA. Differences between (2) and (3) and between (4) and (5) represent DNA-P^{32} estimated by nuclease hydrolysis and acid hydrolysis respectively. Residual P^{32} in cells on slides (3) and (5) must represent phosphoproteins other than nucleoprotein unless there are nucleoproteins not digestable with the nucleases. Since the residual P^{32} following enzyme digestion is often greater than after acid hydrolysis, the enzyme digestions may not remove all nucleic acid.

There are four stages when large amounts of P^{32} are incorporated into the nucleoproteins and phosphoproteins. The first is early leptotene, the second is late diplotene - early diakinesis, the third is just prior to prophase in the microspore and the fourth is the second or third day after the microspore division. At these stages the autoradiographs show that the highest concentration of P^{32} is in the nuclei, but no attempt was made to estimate in a quantitative way the ratio of the isotope in the nucleus to that in the surrounding cytoplasm. Replicate slides were prepared of anthers at these stages for all of the treatments (1) to (5). Since the number of grains remain-

ing after HCl hydrolysis at other stages was either not above background or very low, analysis of the residual P^{32} was not attempted. At leptotene both the enzyme and acid hydrolyses indicate incorporation of P^{32} into DNA. Since RNase removed almost no P^{32} at this stage, incorporation in RNA must be low or absent. HCl hydrolysis removes a much larger fraction of

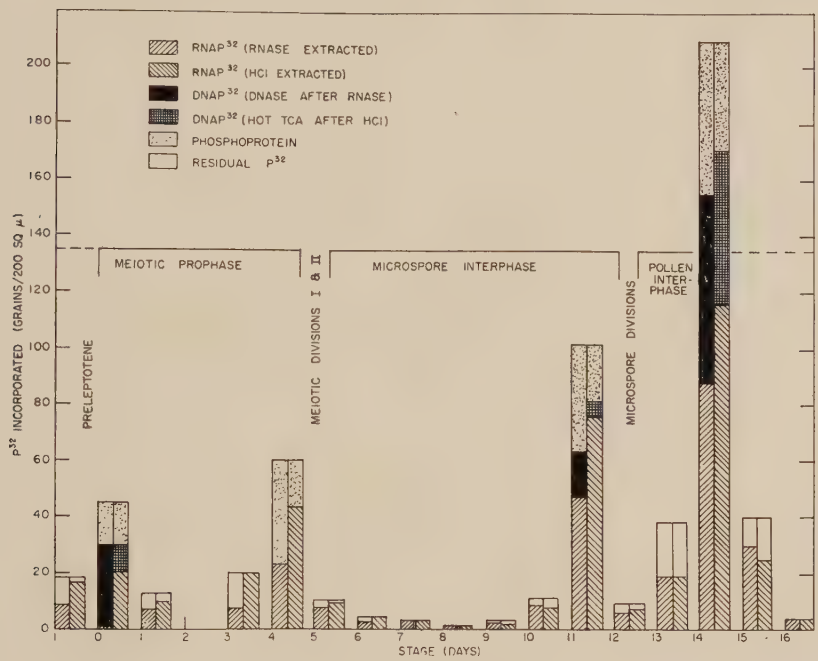

Fig. 1. Nucleic acid and protein fractions incorporating P^{32} during microsporogenesis in *Tradescantia* as determined from autoradiographs.

the P^{32}. This is either a labile DNA or an enzyme-resistant RNA (see Taylor and McMaster [40], for additional data and discussion). Both enzyme and acid hydrolysis indicated that the late diplotene-diakinesis uptake is into RNA and phosphoprotein; no incorporation into, or synthesis of DNA is indicated. This high uptake occurs during the so-called "confused" or diffuse growth stage typical of late meiotic prophase in many organisms [37]. The next period of high incorporation occurs just prior to and during the movement of the nucleus into the central part of the microspore, about 24 to 36 hours before metaphase. P^{32} is incorporated into RNA, DNA and phosphoprotein. The last period of high uptake, which is more rapid than any other, occurs 24 to 36 hours following the microspore division. The recorded grain

counts were made over the generative nucleus. Although the cytoplasm and vegetative nucleus have a high grain count, the generative nucleus is higher at this stage. Part of the uptake is in DNA, but more than half of it is indicated to be in RNA and phosphoproteins.

From the foregoing it can be seen that the P^{32} remaining after HCl hydrolysis is incorporated in both DNA and phosphoprotein. Thus, in the experiment correlating cytophotometric and autoradiographic measurements to be discussed below, the grain count data presented in Table I (column 5) must represent uptake in both fractions.

RNA-incorporated P^{32} varies markedly at different stages of development, but with the exception of the late prophase period just prior to the first meiotic division, the periods of high uptake occur during or immediately succeeding DNA synthesis. Significant amounts of incorporation in the phosphoprotein fraction parallels the uptake in DNA and RNA, but as shown by the behavior at late diplotene-diakinesis may occur without any uptake in DNA. That the residual P^{32} after HCl hydrolysis is probably in phosphoproteins at stages when no net increase of DNA occurs, has been shown in lily anthers [40].

Cytophotometry.—The data on DNA content obtained from measurements of nuclei in sections of a series of buds from a single inflorescence and calculated on the basis of extinction at 470 mμ are presented in Table I, column 3. Although, as mentioned above, no attempt has been made to relate these figures to a known external standard, their significance with respect to chromosome behavior can be better understood if they are compared to an internal standard. According to the concept of "DNA constancy" (for discussion see [28 and 36]), within an organism, a fixed amount of DNA is associated with a haploid chromosome complement. Thus, half the DNA content of a non-synthesizing diploid somatic interphase nucleus represents the base or class amount (C) associated with a single chromosome set. Knowing this amount, it should be possible to predict the DNA content of any cell whose chromosome number is also known, and *vice versa*.

Swift [35] and Moses, Agnew and Sparrow [11] have shown in leaf tissues that the distribution of DNA contents of diploid interphase nuclei fall into a homogeneous unimodal class, the mean and modal values of which represent the "diploid" ($2C$) class. The amount presented in Table I for leaf interphase nuclei may then be taken as the $2C$ amount. On the basis of this figure and the supposed chromosome numbers, the expected amounts for various stages have been calculated and are shown in Table I, column 4. The only time when chromosomes and chromatids can actually be seen and

TABLE I

Relative DNA (Feulgen) content and autoradiographic detection of DNA-P^{32}; *Tradescantia* microsporogenesis.

1	2	3			4	5	
Bud No.	Stage	No. meas.	Amount DNA/ nucleus	± S.E.	Expected DNA/ nucleus	P^{32}† Grains/ 200 μ^2	± S.E.
—	leaf interphase	10	13.0	.78	—	—	
1	pre-early leptotene	10	12.5	.31	13	3	0.5
2	early-mid leptotene	10	19	1.9	13	22	1.0
3	mid-zygotene	10	28	1.7	13–26	3	0.5
5	tetrad (whole cell)	10	26	1.2	26	1	0.3
7	microspore interphase	10	6.6	.22	6.5	0	
8	,, ,,	10	6.2	.36	6.5	0	
9	,, ,,	7	6.1	.40	6.5	0	
10	,, ,,	10	7.6	.65	6.5	2	0.2
11	,, ,,	10	11	1.1	6.5	4	0.6
12	pre-prophase	10	15	1.1	6.5 13	14	0.5
13	pollen (whole cell)	10	13.1	.64	13	4	0.5
14	pollen generative	10	10.4	.83	6.5	19	0.5
	pollen vegetative	7	11.9	1.6	6.5	11*	0.7
15	pollen generative	10	13.7	.78	6.5	15	1.7
	pollen vegetative	10	10.0	.39	6.5	10*	0.7

* It has not been determined how much, if any, of the P^{32} of the vegetative nucleus is in the DNA fraction.

† P^{32} remaining after hydrolysis with *N* HCl, 10 min. at 60°C.

counted is during division and it is then that closest agreement between expected and observed is to be predicted [24]. While no attempt has been made to measure division figures, predictions based on nuclear content immediately before or immediately after division may be made equally well, assuming no further gain or loss of DNA after synthesis has been completed. Thus, for example, post-meiotic telophase (tetrad No. 5) and microspore mitotic telophase (No. 13) amounts agree well with the expected and are respectively about $^1/_4$ and $^1/_2$ of their prophases (No. 3 and No. 12). Such agreement with the predicted is entirely in support of the DNA constancy hypothesis (cf. [6]). The data are further consistent if it is assumed that DNA synthesis may take place before the chromosomes are ever visibly double. It then follows that intermediate DNA values observed between successively higher class intervals must reflect DNA synthesis.

The pre-leptotene nuclei contain the same amount of DNA as the leaf

interphase nuclei (Table I). DNA values increase during leptotene. Synthesis is probably complete before zygotene since incorporation of P^{32} into DNA ceases 12 to 24 hours before zygotene. Autoradiographs of late leptotene and early zygotene nuclei from inflorescences grown the previous 28 hours in P^{32} solution show only low grain counts that may be attributed to phosphoproteins. No change in amount of DNA occurs during the remainder of meiotic prophase. After the two meiotic divisions the quartet of microspores contain the same amount of DNA as the zygotene nucleus; the DNA has neither been gained nor lost in the interval. The DNA content of the individual microspore nuclei remains constant until about 24 to 48 hours before prophase. The incorporation of P^{32} occurs at about the time the nucleus moves to the center of the microspore preparatory to mitosis (see [3], for timing of events). Photometric measurements show the nuclei at pre-prophase (very early prophase of Beatty and Beatty [3]) to have the same amount of DNA or only slightly more than the leaf interphase and pre-leptotene nuclei. After the division, which results in a binucleate pollen grain, both nuclei presumably have equal amounts of DNA. Together they are equivalent to leaf interphase. The generative nucleus, which will divide again in the pollen tube to form sperm nuclei, increases until it has an amount equivalent to the somatic value. The vegetative nucleus, which will disintegrate after the growth of the pollen tube, shows some, but not as marked an increase.

DISCUSSION

The differences reported here in Feulgen dye content at different stages are undoubtedly due to actual variations in DNA content. The possibility of variations due to changes in the stoichiometry of the Feulgen-DNA relationship or to technical errors is slight. In our hands the Feulgen reaction gives good reproducibility and for these experiments all slides were carried through the reaction together. Had the reaction been unstable it is unlikely that the observed DNA classes at such diverse stages as meiotic prophase, telophase, mitotic pre-prophase and post-mitotic interphase would have been in such good agreement with the expected. Furthermore, use of the 2λ method of measuring either avoided or minimized the usual sources of technical error; these probably contributed an over-all variation of not more than a few per cent. One possible source of error, however, was not excluded. Although care was taken to select for measurement only whole nuclei in sections, the inadvertent inclusion of "chipped" nuclei in the sample becomes more probable in stages where nuclear diameter approaches section

thickness as, for example, in early prophase of microspore mitosis. However, that such did *not* occur is shown by measurement of Feulgen smears of this stage from other inflorescences in two experiments which show the DNA content of whole nuclei to be $6.1 \pm .36$ and $6.3 \pm .26$, values equivalent to that of nuclei in sections ($5.8 \pm .42$).

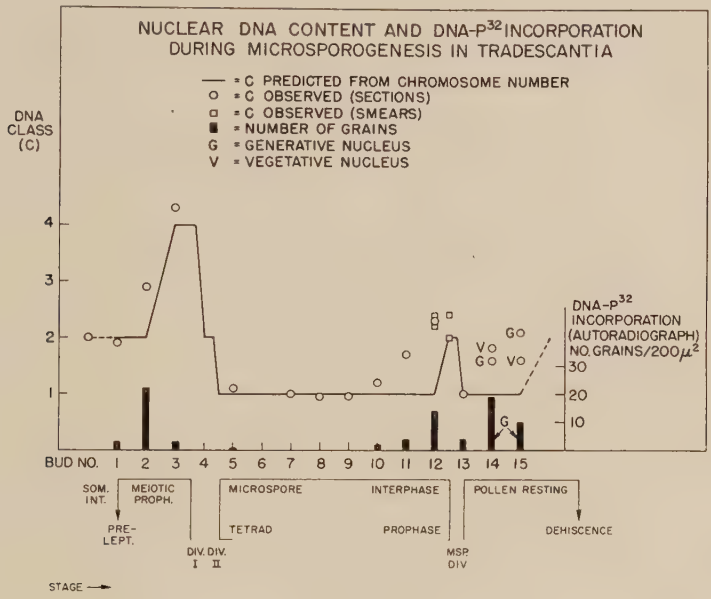

Fig. 2.

The significance of the changes in DNA content becomes apparent when comparisons are made with chromosome behavior. Class values have been calculated by dividing the observed DNA values in Table I by $^1/_2$ the somatic interphase amount. These are graphed together with known and supposed chromosome numbers in Fig. 2. The solid line represents the number of chromosome sets and goes from two to four during meiotic prophase, drops to one after the two reduction divisions, increases to two at microspore prophase and becomes one again in each of the resulting nuclei. The generative nucleus then doubles its set, presumably during tube-division prophase or earlier. Cytologically, nothing can be predicted for the vegetative nucleus which later degenerates.

The generally close correspondence between these and the observed class values is clearly in support of the constancy hypothesis. Only at late microspore interphase and in the binucleate pollen do the observed values depart from the expected. However, since the increments of DNA with time un-

doubtedly reflect synthesis and the amounts eventually correspond to those predicted (with the single exception of the vegetative nucleus), these deviations mean that DNA synthesis occurs before any cytological evidence of chromosome doubling can be observed.

With the exception of the vegetative nucleus, our data on class values are in good agreement with Swift [35] and do not support Bryan [6]. While a fuller discussion of the reasons for this disagreement will be presented elsewhere, it can be said here that although both used the "plug" method, Swift measured at an off-peak absorption wave length and, by thus avoiding the inordinately high extinctions encountered by Bryan, minimized the errors surrounding them. The observations of Schrader and Leuchtenberger [31], in disagreement insofar as they can be compared with ours, are subject to these same errors, as the authors themselves have pointed out. Pasteels and Lison [22], also measuring the DNA content of *Tradescantia*, present no data from which class values may be computed, but their comparative observations agree with Swift's.

The intermediate class value for the vegetative nucleus of the pollen reported here (1.8) suggesting a slow or incomplete synthesis of DNA is not supported by other workers. While the P^{32} results show a slight uptake in these nuclei, they cannot be taken as evidence for synthesis until further experiments have been carried out (as was done for the generative nucleus) to determine whether incorporation was in the DNA or phosphoprotein fraction. That the intermediate DNA value is real was confirmed by additional measurements of slightly earlier stages in smears of other inflorescences; nuclei approximately 12 and 36 hours earlier had class values of 1.4 and 1.3 respectively. However, the possible contribution of varietal or cultural differences cannot be overlooked in accounting for variations in the synthesis pattern. In *Tradescantia*, for example, prior to meiosis, DNA increases during early leptotene but stops at the $4C$ level before zygotene. In *Trillium* [32] the DNA is reported to increase until late pachytene or diplotene, while in *Lilium* synthesis is completed before the beginning of leptotene [40].

Also indicated in Fig. 2 are the DNA-P^{32} incorporation results for the same inflorescence; data are taken from Table I, column 5. It can readily be seen that the three periods of incorporation, meiotic prophase (stages 1 and 2), microspore interphase (stages 11 and 12) and post mitotic (pollen) interphase (stage 14) are also periods of increasing DNA content.

From the first biochemical evidence that P^{32} incorporation in DNA is associated with dividing tissues (e.g. [15, 5, 10]) to more recent reports that

it is related to the increasing DNA content of such tissue (e.g. [33, 2]), there is no indication that phosphorus is incorporated in the DNA except in preparation for mitosis. The autoradiographic results of Howard and Pelc [11], Taylor [39] and Plaut [25] have supported this. These authors have also shown that incorporation occurs during a specific limited time in interphase and have assumed that this represents synthesis. The present evidence (see also the work of Taylor and McMaster on Lily [40]), confirms this period and that of DNA synthesis as being the same.

While these experiments correlate the two processes, it is impossible to tell whether P^{32} incorporation immediately precedes and/or accompanies synthesis of the DNA molecule since autoradiographs and cytophotometric measurements were not made on the same cells, but rather on adjacent sections and some variation in stage of synthesis undoubtedly occurs within an anther. The evidence suggests that the two events are simultaneous, but firmer proof will have to await the results of determination of P^{32} and DNA content of the same cell, which are now in progress.

While it would be very useful to correlate DNA synthesis with physical doubling of the chromosomes, no reliable experimental criterion of the latter is yet available. Cytological observations have given rise to a mass of unresolved disagreements both as to the number of strands comprising a chromosome and the time at which doubling of the number of these strands can first be seen (see [13], for discussion). This is chiefly the result of three major cytological difficulties: the size of the chromatids (chromonemata) approaches the limits of optical resolution; special pretreatments of the material are frequently necessary for observation; the chromosome loses its identity during interphase. As opposed to visual studies, experiments on the time at which chromosomes behave as double entities to breakage by X-rays have the advantage of a more objective experimental approach. However the results are doubly complicated by the difficulty of ascertaining exactly the stage at which the cells were irradiated and the fact that the chromosome may actually be double earlier than the evidence indicates but are insufficiently separated in space to be resolved by the ionizing energy. However, despite these shortcomings such data, particularly those of Sax [30] and his co-workers on *Tradescantia*, provide a useful and functional basis for comparison. While it is well understood that different clones, under different environmental, seasonal and handling conditions may vary in their microsporogenesis schedules, our material behaved quite comparably to that described in the literature; hence a comparison of ours with published data is justified. However, since no experiments have been reported for double-

ness of *Tradescantia* chromosomes to X-rays during meiosis, a correlation can be made only for the two succeeding mitotic divisions.

According to Sax [30], a transition from chromosome to chromatid aberrations occurs 31 to 27 hours prior to metaphase. This corresponds to a period approximately between our stages 11 and 12 when DNA synthesis is being completed.

Bishop [4] reported, for the pollen grain tube nucleus of a similar clone having a comparable timing schedule to ours, that doubleness to X-rays first occurs 3 days prior to dehiscence (2 days after microspore metaphase) and is completed within 24 hours. In our material this corresponds to a stage between our numbers 14 and 15, placing the doubling again at a point where DNA synthesis is being completed.

While this comparison is remarkably consistent in putting the beginning of doubleness to X-rays at or near the completion of DNA synthesis, only an analysis of DNA synthesis and X-ray breakage in the same material can correlate them so closely with any degree of certainty. It is, however, quite reasonable to expect these two events to be closely allied and the evidence clearly shows this. There can be little doubt that DNA synthesis and chromosome doubling occur close together and hence must be intimately interrelated.

One other point of interest emerges from these results. Several workers [e.g. 21, 41] have attempted to assign a generalized fixed period of the division cycle to synthesis. To the contrary, our data would seem to show that for the three divisions which were preceded by synthesis, different parts of the cycle of all three were involved. Thus it occurred in early prophase of meiosis, late interphase of the microspore division and mid-interphase of the tube division. By way of comparison, Howard and Pelc [12] showed in *Vicia* root tips that DNA synthesis occupies about a quarter of the interphase and begins about half-way through. It seems preferable to think of the timing of synthesis as being characteristic for a particular cell type or tissue and as being dependent on other factors than a rigid scheduling of the "resting" phase. It also follows that the succeeding prophase is not necessarily obliged to, but may commence immediately upon completion of synthesis. Since cells may remain "resting" with their doubled content of DNA for varying lengths of time before visible prophase begins, it cannot be synthesis that triggers the process of division, although, except for the second meiotic division, the cells cannot divide until synthesis is completed. DNA synthesis, therefore, appears to be a necessary rather than an initiating process for mitosis.

SUMMARY

1. DNA-P^{32} incorporation and DNA content of nuclei in various stages of *Tradescantia* microsporogenesis were determined by means of autoradiographs and cytophotometric measurements of the Feulgen reaction.

2. The DNA content was found to follow that predicted on the basis of the "constancy hypothesis".

3. P^{32} was incorporated in the DNA fraction only during those periods when the DNA content was in the process of doubling.

4. DNA synthesis occurs at different specific periods characteristic for each of the succeeding sporogenic divisions: early prophase of the first meiotic division, late interphase of the microspore division and mid-interphase of the pollen division. There can thus be no generalized period in the division cycle when DNA synthesis occurs; it seems to depend on the nature of the division.

5. From correlations with published X-ray breakage data, it appears that completion of DNA synthesis occurs about the time chromosomes first behave as double entities to X-rays.

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AMINO COMPOUNDS AND ETHANOLAMINE PHOSPHORIC ACID OF THE GRASSHOPPER EGG

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Although the grasshopper is currently being used in cytological, physiological, and radiological studies in many laboratories, little is known about its chemical composition and methods of culturing it. Drilhon and Busnel [19] studied the amino acid content of the egg of the silkworm, *Bombyx mori*, at several stages of development. Slifer [45] reported the quantity of fatty acids found in the egg of *Melanoplus differentialis*, a grasshopper. The quantity and distribution of nitrogen [50] and of phosphorus [49] were also determined for *Melanoplus*. Lu and Bodine [34] also reported on the distribution of phosphorus and its changes throughout development. None of these data, however, have been applied to the development of a culture medium for the maintenance of the grasshopper embryo outside of the egg. At the present a culture medium, which has been used, consists of inorganic salts, glucose and some grasshopper egg yolk [14]. Such a medium does not maintain cell division at a constant rate for more than a few hours. The degenerative changes observed in old cultures are similar to those known to be caused by hypertonic media. Larval insect blood has a very high concentration of amino acids, which may contribute considerably to the osmotic pressure of the blood [41]. The major portion of the anions of insect blood is composed of free amino acids while unbound chlorides are present in low concentration [41]. Determination of the nature and concentration of these osmotically active organic anions seems essential if a more satisfactory culture is to be devised.

MATERIALS AND METHODS

Preparation of extracts.—Eggs of the grasshopper *Chortophaga viridifasciata* De Geer at the stage equivalent to 14 days development at 26°C were used for chromatographic determination of amino acids. In a study on the effect of the stage of development on the amino acid composition, both younger and older eggs were used. Approximately 80 eggs were opened in 2 ml of 80 per cent ethanol and the

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entire contents thoroughly dispersed in the solution. Another 3 ml of 80 per cent ethanol was added to make the final volume 5 ml per 80 eggs. The contents of the eggs were extracted by allowing them to stand in the alcohol at room temperature for 15 to 20 minutes, after which the precipitated yolk, embryonic cells, and denatured protein were removed by centrifugation for 5 minutes in an International Clinical Centrifuge. The supernatant was divided into approximately equal fractions, each of which contained an amount of material equivalent to that extracted from 10 eggs. Each fraction was dried with a jet of clean filtered air at room temperature and then refrigerated (usually for no longer than one week) until used.

SPECIAL TREATMENT OF EXTRACTS

Hydrolysis of extracts was accomplished in 6 *N* HCl at 100°C for 24 hours in sealed glass tubes. The hydrochloric acid treated extracts were evaporated to dryness at room temperature with an air jet. The oxidation of sulphur-containing amino acids was accomplished by the method outlined by Dent [18]. One half ml of distilled water was added to the extract in addition to 0.2 ml of 30 per cent hydrogen peroxide (superoxol). After one minute another 0.2 ml of superoxol was added to the extract with 0.6 ml of 0.02 per cent ammonium molybdate solution. The solution was incubated at 60°C for 20 minutes after which it was evaporated to dryness.

Preparation of chromatograms. –Extracts were redissolved in approximately 20 lambda of 80 per cent ethanol. Higher concentrations of ethanol were avoided because they reduce the solubility of some of the compounds and cause their loss by precipitation. The solution was applied to 18 by 22 inch Whatman No. 1 filter paper. The techniques used were those outlined by Dent [18], except that ethylene diamine was not present during the lutidine runs. Redistilled phenol saturated with distilled water was used as the first solvent. The second solvent was either lutidine or butanol. Lutidine consisted of the organic rich phase of a mixture of lutidine, collidine, and distilled water in the ratio of 3:1:1. Butanol consisted of a mixture of equal volumes of each of the following two solutions: an 89 to 6 volume to volume ratio of butanol to distilled water, and a 62 to 79 volume to volume ratio of proprionic acid to distilled water. Color development was obtained by treating chromatograms with a 0.1 per cent solution of ninhydrin reagent in 95 per cent ethanol and heating to 100°C for about 5 minutes.

Preparation of chromatographic maps.—The position of each of 28 different crystalline compounds (usually commercial preparations) was determined on two dimensional chromatograms. In these chromatograms the first solvent was phenol and the second was lutidine in one set and butanol in the other set. Thus two standard chromatographic maps were established on which the relative positions of the 28 compounds were demonstrated for each set of solvents. Fig. 1B shows their relative positions when phenol and lutidine are the solvents and Fig. 1IB shows them when phenol and butanol are used.

Preparation of autoradiograms. –Adult grasshoppers were fed barley grass containing radioactive phosphorus[1] in order to incorporate P^{32} into the eggs. The

[1] The radioactive barley grass was kindly supplied by Dr. N. E. Tolbert, Biology Division of the Oak Ridge National Laboratory.

eggs were allowed to develop to the 14 day stage and were extracted. The extracts were chromatographed in the usual manner. After color development was obtained, the dry chromatogram was placed in darkness against Kodak X-ray film for one week after which the film was developed. The blackened areas which indicated the location of the compounds containing P^{32} were compared with the location of the ninhydrin positive spots on the chromatogram.

Hydrolysis of ethanolamine phosphoric acid.—The eluate from the ethanolamine phosphoric acid region of six chromatograms were combined, dried, and redissolved in 1 ml of distilled water. To one half of this solution several crystals of alkaline phosphatase[1] and magnesium chloride were added. This was incubated at room temperature for 24 hours. The other half was incubated at room temperature for six hours with 20 lambda of a 1 mg/ml of Polidase-S solution (Schwartz Laboratories). These enzyme treated eluates were chromatographed to detect possible chemical changes.

Specific color reactions.—The presence of citrulline was demonstrated by spraying the chromatograms with a 1 per cent solution of *p*-dimethylaminobenzaldehyde in 1 *N* HCl [23]. The reagent reacts specifically with ureide forming compounds to produce a yellow color. It also reacts with tryptophane producing a brownish-orange color on phenol and butanol chromatograms. To definitely distinguish arginine from other basic amino acids, the Sakaguchi reaction [9] was applied to the combined eluates of the arginine region of two chromatograms. The combination of eluates from two chromatograms was necessary because of the low sensitivity of this reaction as compared to the ninhydrin reaction with arginine. To 2 ml of eluate 0.5 ml of 10 per cent NaOH was added with five drops of 0.1 per cent α-naphthol in 95 per cent ethanol. After 5 minutes 8 to 10 drops of NaOCl solution (Chlorox) was added and a cherry red color developed.

The Haynes-Isherwood phosphate test [7] was applied to chromatograms to determine the position of phosphorus containing compounds. Owing to the remarkable stability of the phosphate bond in ethanolamine phosphoric acid however, the location of this compound could not be demonstrated by means of this test.

RESULTS

Identification of amino acids.—Fig. IA shows the relative positions of the ninhydrin positive spots on chromatograms of egg extracts chromatographed in phenol and lutidine. Fig. IIA shows the same for those extracts chromatographed in phenol and butanol. A comparison of Figs. IA with IB and of IIA with IIB indicates that certain of the ninhydrin positive spots on chromatograms of egg extracts can tentatively be identified by the similarity of their relative positions to those of known compounds on the chromatographic maps. The identity of the amino compounds listed in Table I is

[1] The alkaline phosphatase was obtained from Dr. N. E. Tolbert, Biology Division of the Oak Ridge National Laboratory.

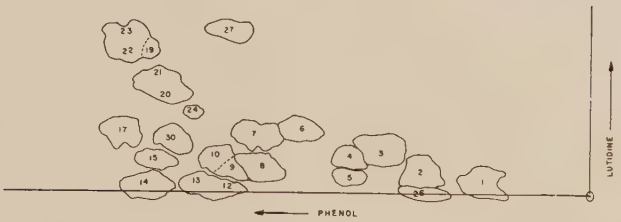
TABLE I

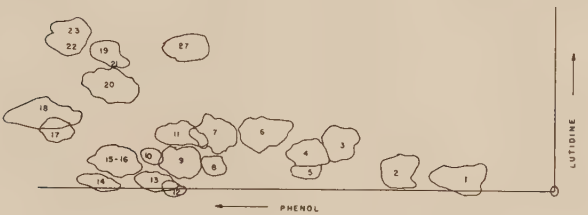
Ninhydrin-sensitive compounds found in the egg of *Chortophaga viridifasciata* which may be considered identified without further proof by the correspondence of their relative positions and colors to those of known compounds on prepared chromatographic maps of crystalline standards.

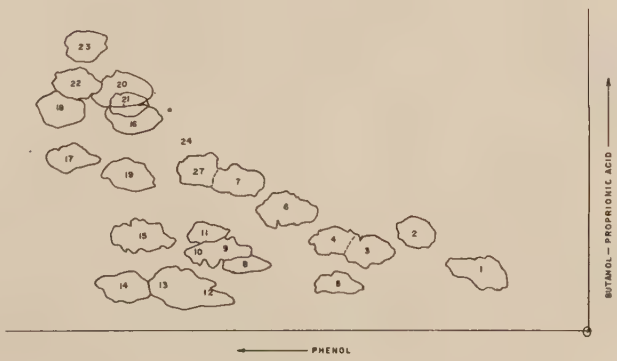
Phenol-lutidine chromatograms			Phenol-butanol chromatograms		
Spot no.	Compound	Color	Spot no.	Compound	Color
7	β-alanine	purple	7	β-alanine	purple
14	arginine	purple	14	arginine	purple
5	asparagine	tan	5	asparagine	tan
1	aspartic acid	blue	1	aspartic acid	blue
2	glutamic acid	purple	2	glutamic acid	purple
4	glycine	purple	4	glycine	purple
17	proline	yellow	23	leucines	purple
3	serine	purple	15	methionine sulphoxide	purple
6	threonine	purple			
27	tyrosine	grey	22	phenylalanine	purple
			17	proline	yellow
			3	serine	purple
			6	threonine	purple
			19	tryptophane	grey-purple
			27	tyrosine	grey

assumed to be correct without further experimental proof because there are no other commonly known compounds which chromatograph to the same region and produce spots of the same shape or color. Although β-alanine tends to move to the same position as citrulline on chromatographic maps, the presence of β-alanine (spot 10) may still be demonstrated on chromatograms of egg extracts by the bright blue appearance of the β-alanine fraction of the composite spot. Citrulline produces a purple color in contrast to the bright blue of β-alanine and is demonstrated by a specific color reaction (see above).

Acid hydrolysis of extracts causes the destruction of certain compounds and changes in others. This consequently causes the disappearance of some spots and the relocation of others. After such treatment glutamine (spot 8) is destroyed: the majority of it being broken down to glutamic acid (spot 2). This results in an increase in the size and intensity of the glutamic acid spot. The amide asparagine (spot 5) and the very acid labile amino acid tryptophane (spot 19) are usually completely destroyed. Spot 26 becomes less







Figures I A, I B, II A, II B.

1. aspartic acid
2. glutamic acid
3. serine
4. glycine
5. asparagine
6. threonine
7. alanine
8. glutamine
9. citrulline
10. β-alanine
11. hydroxyproline
12. ornithine
13. lysine
14. arginine
15. methionine sulphoxide
16. γ-amino butyric acid
17. proline
18. pipecolic acid
19. tryptophane
20. valine
21. methionine
22. phenylalanine
23. leucines
24. methionine sulphone
25. lanthionine
26. ethanolamine phosphoric acid
27. tyrosine
28. cystathionine
30. unknown

intense in color and smaller in size, and a pale ninhydrin positive spot appears between proline (spot 17) and phenylalanine (spot 22).

When chromatograms of egg extracts are treated with the ureide sensitive reagent, *p*-dimethylaminobenzaldehyde, a yellow spot appears in the same position as the 9 region of the compound 8–9–10 spot, indicating that citrulline is present. Another faint yellow spot appears in a region directly beneath the threonine area. However it does not correspond directly with the position of threonine or any other ninhydrin positive spot and is therefore not likely to be an amino acid. A brownish-orange spot is produced in the region of spot 19 indicating that it is tryptophane [9].

After hydrogen peroxide oxidation spot 15 (methionine sulphoxide) disappears completely and spot 24 appears above and to the left of alanine (spot 7). Since spot 24 corresponds with methionine sulphone on the chromatographic maps, spot 15 is probably methionine sulphoxide. Valine (spot 20) and methionine (spot 21) both chromatograph to the same position on chromatographic standards. The spot found on phenol-butanol chromatograms of egg extracts is composed of two distinct shades of purple before peroxide oxidation: one more blue than the other. The treatment causes a slight decrease in the size of the 20–21 spot and the disappearance of the bluer shade. Both of these facts indicate that this spot contains, besides valine, a small amount of methionine, which has been oxidized to the sulphone. If it had consisted wholely of methionine, the entire spot would have disappeared after peroxide oxidation of the egg extract.

The 12–13 spot can not be separated into two discrete spots with the different sets of solvents used. Thus it may have been produced by lysine or ornithine alone, or by a mixture of both compounds. The corresponding spot on the chromatographic map indicates that even with pure amino acids these compounds are not easily separated by paper chromatography.

Spot 30 (in Fig. IA) is present only in phenol-lutidine chromatograms of egg extracts. It is either distroyed or co-chromatographs with another compound in these chromatograms. According to the chromatographic standard map by Dent [18], its relative position is similar to that of either ε-hydroxy-α-amino caproic acid or α-amino-iso-butyric acid. It does not move far enough in phenol nor in lutidine to be α-amino-*n*-butyric acid.

Identification of spot 26.—The location of this spot, when compared with those on Dent's map [18], suggests three possibilities: lanthionine, cystathionine, and ethanolamine phosphoric acid (EPA). Chromatographic maps prepared with crystalline samples of aspartic and glutamic acids in addition to these three compounds indicates that, by using only their relative

TABLE II

A. Amino compounds identified as present in the egg of *Chortophaga.*

Amino compound	Evidence for its identity		
	Relative position	Color of spot	Other tests
α-alanine	x		
β-alanine	x	x	
Arginine	x		Sakaguchi reaction
Asparagine	x	x	Acid labile
Aspartic acid	x	x	
Citrulline	x		Ureide test
Ethanolamine phosphoric acid	x		Acid and enzyme hydrolysis, autoradiograms
Glutamic acid	x		Hydrolysis of glutamine augments this spot
Glycine	x		
Leucine	x		
Methionine	x	x	Peroxide oxidation
Lysine and/or ornithine	x		
Phenylalanine	x		
Proline	x	x	
Threonine	x		
Tryptophane	x	x	Reaction with *p*-dimethylaminobenzaldehyde, acid labile
Tyrosine	x	x	
Valine	x		

B. Amino compounds commonly found in insects but not detected here.

α-amino-*n*-butyric acid
γ-amino butyric acid
Cysteine or cystine
Histidine
Homocysteine or homocystine
Hydroxyproline
Taurine

positions as the distinguishing criterion, spot 26 is caused by the presence of EPA.[1] Acid hydrolysis of egg extracts in 6 *N* HCl at 100° C for 24 hours

[1] The standard was prepared from the barium salt of ethanolamine phosphoric acid kindly provided by Dr. Jorge Awapara, M. D. Anderson Hospital, Houston, Texas.

decreases the size and intensity of spot 26 (but does not destroy it completely) and a faint spot appears in a position between proline and phenylalanine. The appearance of the new spot at the location corresponding to that of synthetic ethanolamine indicates that part of the compound in spot 26 is composed of ethanolamine.

Phosphatase-treated eluates of spot 26 produce an intense spot on the chromatograms in the same location as that of synthetic ethanolamine on the chromatographic standard, which indicates again that the derivative of the compound at the location of spot 26 is ethanolamine. That it acts as a substrate for the enzyme, alkaline phosphatase, is evidence that it is a phosphate ester. Further, autoradiograms show that spot 26 is radioactive when chromatograms are produced from extracts of eggs containing radioactive phosphorus.

Amino acid content at different stages of development.—Comparison of chromatograms made from extracts of eggs of different ages (7 days, 14 days, and three weeks developments at 26° C) revealed that the older stages contained higher concentrations of free amino acids than the earlier stage. All of the ninhydrin positive compounds detected were found on the chromatograms of all of the stages of development studied. As development progressed, no new ones appeared and none previously present disappeared. Also when the amino acid concentration increased in the later stages, the concentration of every spot increased proportionately.

DISCUSSION

Although many amino compounds were detected, only a few are of special interest. They indicate certain facts about the metabolism of the grasshopper egg and their presence is difficult to explain. These compounds are ethanolamine phosphoric acid (EPA), citrulline, and methionine sulphoxide.

Occurrence and possible significance of EPA.—When Outhouse [37, 38] first discovered that an acid soluble fraction of bovine tumor phosphate contained ethanolamine phosphoric acid, he was unable to find it in normal tissues and believed that its presence was peculiar to tumors. Colowick and Cori [16] were able to extract the compound from normal intestinal mucosa of rabbits and pigs. Since then EPA or 'underglutamic'[1] has been found in

[1] This is the name found in the literature for a compound which chromatographs to beneath glutamic acid on two dimensional chromatograms and has been shown in many instances to be ethanolamine phosphoric acid.

the normal tissues of numerous vertebrate organisms [1, 3, 4, 5, 6, 12, 13, 31, 53]. This is the first report of the presence of EPA in the grasshopper although it has been found in another invertebrate, the sea urchin [33].

EPA or 'underglutamic' has been found in high concentrations in eggs, embryos, and rapidly growing tissues [8, 20, 21, 25, 29, 42]. It has been found in tumors [28, 37, 38, 44] but not always in high concentration. The level of EPA concentration in tumors apparently varies with the type tumor. Sauberlich and Bauman [43] indicated that some types of tumors (such as mammary fibrosarcomas) contain large amounts of intercellular material which decreases the concentration of the cellular protoplasm. These authors consider the chemical nature of these tumors to be similar to that of normal tissue diluted with connective tissue. Thus even if the EPA concentration in the tumor cells was higher than that of normal cells, the concentration of EPA in the whole tumor (cells plus fibers) would appear closer to the concentration found in normal tissue or lower. Besides the high concentration of amino acids usually found in rapidly growing tissues, Rouser and Tishkoff [42] found EPA and another nitrogen-containing phosphorylated compound in rabbits. They suggested that the presence of phospholipid fragments might be characteristic of rapidly growing tissues. The presence of EPA in the grasshopper egg raises the question of its role in development. Several clues may be obtained from vertebrate physiology where EPA and to a greater extent ethanolamine have been studied. Ethanolamine and EPA are present in phospholipids and are intermediates in their synthesis. EPA occurs in the cephalin, phosphatidylethanolamine [6, 27, 29, 30, 31] and in combination with glycerol to form glycerylphosphorylethanolamine (GPE) [2, 12, 13]. Chargaff and Keston [15] tried to show that the EPA molecule was incorporated intact into cephalins by injecting P^{32} labeled EPA into rats. The results indicated that EPA was dephosphorylated before it could enter the cell. Thus there were no indications as to whether or not EPA could be directly incorporated into cephalin if it got into the cell intact. Ethanolamine is readily converted into choline in the rat. The former is methylated by the transfer of methyl groups from methionine. Monomethyl- and dimethylethanolamine are probably intermediates in the synthesis of choline for both are good precursors [26, 51, 52].

The grasshopper has a very active lipid metabolism. Fatty acids comprise 20 per cent of the dry weight of the egg of *Melanoplus differentialis* and this quantity is reduced to one half its original amount by the end of embryonic development [45]. Bodine [10] and Hill [24] eliminated the possibility that proteins or carbohydrates are a major energy source in the grasshopper

egg. The R.Q. averages 0.71 except during the first day of development [11]. Boell also calculated that, during the entire course of development of the grasshopper egg, 72.5 per cent of the oxygen consumed can be accounted for on the basis of the oxidation of fat known to disappear from the egg.

Origin of EPA.—Does the origin of EPA in the grasshopper egg lie in the catabolism of the phospholipids containing it? Stetten [46, 47] suggested that it was not produced by the hydrolysis of phosphatidylethanolamine (PTE). Ansell and Dawson [1] through the use of P^{32} showed that the specific activity of the EPA was too great for it to have been produced by the hydrolysis of PTE. Norman and Dawson [36] arrived at a similar conclusion. Ansell and Dawson [1] also demonstrated the synthesis of EPA in minced rat brain. Ansell and Norman [2] demonstrated that GPE is not a product of the catabolism of PTE and that GPE does not lie in the main synthetic pathway of PTE. They also demonstrated the phosphorylation of ethanolamine to EPA *in vitro* and the synthesis *in vitro* of GPE. This suggests that EPA is probably a direct precursor of GPE.

Since EPA is not produced by the catabolism of PTE or GPE, it must have some other origin. Nord [35] suggested that anaerobic bacteria could produce ethanolamine by the decarboxylation of serine. Folch [22] suggested that the cephalins containing ethanolamine were artefacts formed by the post mortem decarboxylation of the serine element of some cephalins. The pathway of synthesis of ethanolamine has been demonstrated *in vivo*. Stetten [46, 47] showed that N^{15} labeled glycine was transformed to ethanolamine in rats. It was also shown by Stetten [48] that labeled serine was decarboxylated to ethanolamine and the latter incorporated into phospholipids. Since ethanolamine itself was not isolated in his study, the actual intermediate formed could have been a phosphorylated compound such as EPA. Levine and Tarver [32] injected rats with serine-β-C^{14} and recovered some of the C^{14} in the form of $C^{14}O_2$, indicating the formation of a two carbon compound, perhaps glycine. However, some of the C^{14} was found in ethanolamine indicating the decarboxylation of serine.

Free ethanolamine could not be detected on the chromatograms of egg extracts. This suggests that EPA is not produced by the phosphorylation of ethanolamine in the grasshopper egg. It may have been produced by the decarboxylation of a phosphorylated precursor, such as serine phosphate. The precursor could be produced by the transamination of a phosphorylated organic acid such as 3-phosphoglyceric acid. Delluva [17], working with *E. coli*, suggested that serine must originate from a three carbon fragment derived from glucose metabolism. However, serine phosphate was not

detected on the chromatograms of egg extracts. Thus the origin of EPA in the grasshopper egg is not clear.

Metabolism of EPA and nitrogen elimination.—Ethanolamine is metabolised in several ways when injected intraperitoneally into rats. About 10 per cent is oxidized to CO_2 in twenty-four hours. Some is converted into serine and choline, and to some slight degree to glycine [40]. When fed to pigeons it is deaminated, oxidized, and reaminated to form glycine. However, 75 per cent of the radioactive carbon of the ethanolamine administered ends up in uric acid [54]. When $C^{13}H_2OH$–$C^{14}H_2NH_2$ is fed to pigeons, the C^{14} carbon becomes carbon 4 and C^{13} becomes carbon 5 of uric acid. Both carbons 4 and 5 of uric acid are derived from glycine. Thus ethanolamine is intricately involved in the metabolism of both serine and glycine, and nitrogen elimination in those forms which produce uric acid.

Citrulline and nitrogen elimination.—Among the ninhydrin positive substances in the insect egg listed in Table II, the presence of citrulline was unexpected. The major established function of this amino acid is thought to be its part in the production of urea, in vertebrates which eliminate nitrogen in this form, through its role in the arginine-citrulline-ornithine cycle. The grasshopper embryo eliminates nitrogen in the form of uric acid. The blood of some larval insects contains a very small quantity of urea [41]. Arginine and citrulline were demonstrated in the egg of *Chortophaga* but the presence of ornithine is uncertain. Thus there is some doubt as to the role of citrulline here.

Methionine and other sulphur amino acids.—The methionine sulphoxide found is probably an artefact produced during the preparation of the extracts. The methionine originally present was probably oxidized by the oxygen present in the air of the air jet used in the drying process. Methionine or its oxidized form is the only sulphur amino acid or amine which is found to be present in sufficient concentration to be detected. If methionine is active in the production of choline, the presence of homocysteine (or homocystine) would be expected. Neither of these compounds is detected on the chromatograms of egg extracts. Other workers [19, 39] found cysteine (or cystine) and taurine in the insect.

SUMMARY

The presence of 16 or 17 amino acids, amines, ethanolamine phosphoric acid (EPA), and one unknown compound has been demonstrated in the egg of the grasshopper *Chortophaga viridifasciata* De Geer by means of

paper partition chromatography. The role of citrulline in nitrogen elimination in this organism is questionable. EPA may be active in nitrogen elimination. Methionine is the only sulphur amino acid detected. Ethanolamine is metabolically related to serine and glycine. EPA may have the same metabolic relationship to these amino acids as does ethanolamine. The presence of EPA and a high lipid metabolism are probably characteristic of rapidly growing tissues. EPA may be necessary for the maintenance of tissue cultures of the grasshopper embryo.

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THE ROLES OF THE NUCLEIC ACIDS AND FREE NUCLEOTIDES IN CHICK EMBRYONIC EXTRACT ON THE GROWTH OF HEART FIBROBLAST[1]

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In 1946 Fischer [2] distinguished between the high molecular weight and the diffusible constituents of embryonic extract (*EE*). He found that dialyzed extracts failed to support growth of fibroblasts, but that this effect of dialysis was partially reversed by the addition of a mixture of amino acids [3].

Subsequently, three groups of investigators [5, 13, 14] have studied the role of ultrafiltrates, or of alcoholic extracts of *EE*, on proliferation of fibroblasts *in vitro*. These solutions were shown to contain a great variety of compounds, including amino acids, purines, pyrimidines, sugars, and coenzymes. There appears to be general agreement that these low molecular compounds are essential for growth.

However, the importance of the proteins and nucleic acids of *EE* has also been established. Fischer [4] described a nucleoprotein fraction, termed "embryonin", which promoted growth of cultures; later work by Katsuta and Takaoka [7] and Kutsky [8] has emphasized the essentiality of nucleic acids in the nutrient media.

Recent studies conducted in our laboratory have been concerned with the correlation of growth, as measured by increase in desoxyribose nucleic acid content, with the incorporation of labeled adenine and thymidine into nucleic acids in roller tube tissue cultures [9]. The present paper is a continuation of these experiments, but with emphasis on the effect of fractionation of the *EE* nutrient medium on its growth-promoting properties. Various chemical analyses have been performed in order to characterize the major fractions.

MATERIALS AND METHODS

The following nutrient fractions were prepared:

Dialyzed embryonic extract (*DEE*).—50 per cent *EE* [9] was dialyzed in a cellophane tube against three changes of Tyrode's solution, for a total of 48 hours at 5°.

[1] Aided by a grant from the National Institutes of Health, U.S. Public Health Service.

Perchloric acid-soluble fraction (*PS*).—To 50 per cent *EE* was added an equal volume of cold 0.6 *N* perchloric acid. The precipitated protein was centrifuged and extracted again with one volume of cold 0.2 *N* $HClO_4$. The combined supernatant fluids were carefully neutralized to pH 7.0 by the addition of 5 *N* KOH at 0°C. The resulting precipitate of $KClO_4$ was removed as completely as possible by centrifuging in the cold. The supernatant liquid was then evaporated to dryness *in vacuo*. The dry residue was dissolved in the minimal volume of water, and the undissolved $KClO_4$ was again removed by centrifugation. The clear solution was finally reconstituted with water to the original volume of the 50 per cent *EE*, and sterilized by filtration through UF-Pyrex sintered glass filters into "Plasma-Vac" bottles. In certain experiments, isotopically-labeled *PS* and *DEE*, containing purine-C^{14} nucleotides and nucleic acids, were prepared from radioactive embryos (fertile eggs injected with adenine-8-C^{14}) [10].[1]

Heat-stable supernatant (*HS*).—50 per cent *EE* was heated at 100°C for 5 minutes. The coagulum was centrifuged, and the clear supernatant solution was decanted and retained.

Ethanol fractionation.—One volume of *HS* was mixed with two volumes of 95 per cent ethanol, and kept at −20°C for 4 hours. The resulting alcohol-insoluble precipitate (*AI*) was separated by centrifugation, and the supernatant alcohol-soluble phase (*AS*) was decanted. Each fraction was freed of alcohol by lyophilization. Then *AS* was reconstituted with water to the original volume of *HS*, while *AI* was similarly reconstituted with Tyrode's solution.

Tissue culture procedures.—Previously described methods [9, 10] were used for explantation and cultivation of 13-day old chick heart fibroblasts, and for determining the nucleic acid content and radioactivity of the cultures. Besides the standard *EE* medium, combinations of the nutrient fractions discussed in the preceding section, were used. Changes in the composition of the media at successive stages of cultivation were determined by chemical analysis (described in the next section), and by C^{14} measurements in those cases in which labeled adenine, nucleotides or nucleic acids were employed [10].

Analysis of nutrient fractions.—The orcinol [11] and diphenylamine [1] reactions were used for the quantitative determination of RNA and DNA, respectively. Likewise non-polynucleotide ribose compounds were determined by the orcinol method.[2] Total nucleoprotein content [9] and the sum of amino acids plus peptides [15] were also measured.

RESULTS AND DISCUSSION

In Table I the main difference observed between the original *EE* and the dialyzed preparation (*DEE*) was in the concentration of ribose compounds. These fell from 16 to 1.7 mg per 100 ml of 50 per cent *EE*. The levels of nucleoprotein, RNA, and DNA did not decrease markedly. Most probably

[1] Allocated by the U.S. Atomic Energy Commission, Oak Ridge, Tennessee.

[2] Desoxyribose nucleotides could not be measured accurately because of their low concentrations and the insufficient sensitivity of the diphenylamine reagent.

TABLE I

Characterization of *EE* fractions.

Fraction	Concentration in mg per 100 ml of 50 per cent *EE*			
	Nucleo-protein	DNA	RNA	Non-polynucleotide ribose
Original *EE*	1360	5.5	55.8	16
Dialyzed (*DEE*)	1260	4.2	41.1	1.7
Perchloric acid soluble (*PS*)	0	0	0	16
Heated supernatant (*HS*)	145	1.5	9.0	14
Alcohol-soluble (*AS*)	0	1.5	0	12
Alcohol-insoluble (*AI*)	210	0	8.5	1.6

the ribose compounds lost during dialysis were chiefly nucleotides (in the light of data to be presented later).

Further confirmation of the extensive removal of purine and pyrimidine compounds was the shift in the ultraviolet absorption maximum from 258 to 270 mμ, upon the dialysis of *EE* (Fig. 1).

It may be seen (Table I) that the perchloric acid completely removed nucleoprotein, RNA, and DNA, while heat treatment of *EE* removed most, but not all of these substances. Ribose nucleotides were retained in both *PS* and *HS*. The latter fraction seems most nearly equivalent to the diffusible components of *EE*, in that the sums of *DEE* plus *HS* values for nucleoprotein, DNA, RNA, and ribose compounds were approximately equal to the corresponding values for the original *EE*. Similarly, the *AS* plus *AI* values approximeted those for *HS*, as was to be expected. It was found that the nucleoprotein and RNA remaining after heat treatment were precipitated by the addition of ethanol, while the residual DNA and most of the ribose compounds remained in solution.

Fig. 2 shows that fractions *PS*, *HS*, and *AS* had almost identical absorption maxima (255 mμ), and that these were only slightly displaced from that of the original *EE*. On the other hand, *AI* had its peak at 263 mμ, and its curve resembled more nearly that of *DEE*.

As reported in an earlier paper [9], the DNA content of chick fibroblasts, cultivated in 25 per cent *EE*, increases rapidly, after the initial fall associated with explantation. In Fig. 3 it is of interest that the combination of *DEE* and *HS* gave the same increase in tissue DNA at 6 days, as that obtained with the complete *EE*. A lesser response was obtained when *PS* replaced

TABLE II

Utilization of protein and nucleic acid constituents of *EE* during cultivation of heart tissue. For each time interval the changes in the *EE* were calculated in terms of the original medium, since the later was renewed every three days.

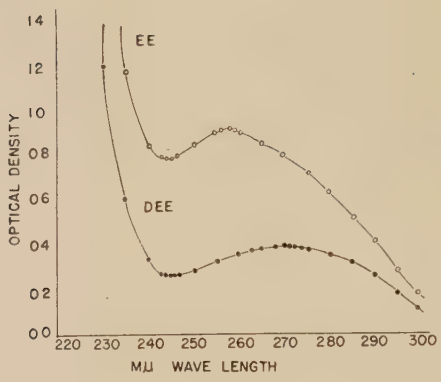
Period of cultivation	Concentration in mg per 100 ml of 25 per cent *EE*				
	Nucleo-protein	DNA	RNA	Non-polynu-cleotide ribose[1]	Amino acids and peptides[2]
Control *EE* (3 days without tissue)	623	2.7	27.2	7.25	0.70
0–3 Days	606	2.2	23.2	6.10	0.65
Decrease	17	0.5	4.0	1.15	0.05
3–6 Days	602	1.8	22.0	6.20	0.63
Decrease	21	0.9	5.2	1.05	0.07
6–9 Days	596	1.8	21.6	6.05	0.625
Decrease	27	0.9	5.6	1.20	0.075

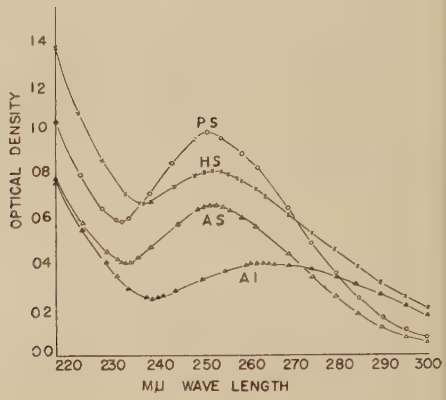
[1] Soluble in cold perchloric acid.
[2] m*M* per 100 ml, determined with trichloracetic acid filtrates.

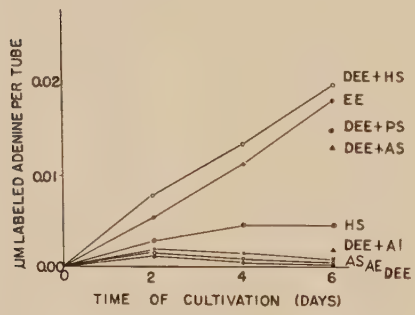
HS. The former fraction, unlike *HS*, was completely devoid of polynucleotides. Also, traces of perchlorate in *PS* may have been inhibitory, *AS* plus *DEE* was still less satisfactory. It is apparent that neither *AS* nor *DEE* alone, nor *DEE* plus *AI*, were able to cause an increase in the DNA content of the cultures.

Kutsky [8] reported that the nucleoprotein fraction of *EE* exerts a stimulating effect in the production of heart fibroblasts. Likewise, Nishioka and Ibuka [12] concluded that highly-polymerized RNA promotes the growth of cultures. In the present study, it was found that both *HS* and *AS* contain relatively large amounts of ribose compounds and amino acids. Thus the inability of these fractions to support tissue growth must be due in large measure to deficiencies in protein, RNA, and (probably) DNA. This conclusion agrees with that of other workers [4, 7].

The utilization of both nucleic acid and protein components of the nutrient medium by the heart cultures is demonstrated in Table II. If the decreases in RNA and DNA in each of the time intervals are subtracted from the corresponding nucleoprotein values, the quantities of protein utilized can be seen to range from 12.5 to 20.5 mg. These values may be compared with the uptakes of the amino acid-peptide fraction, which range from about 8 to 11 mg (taking one m*M* as 150 mg). The extensive contri-







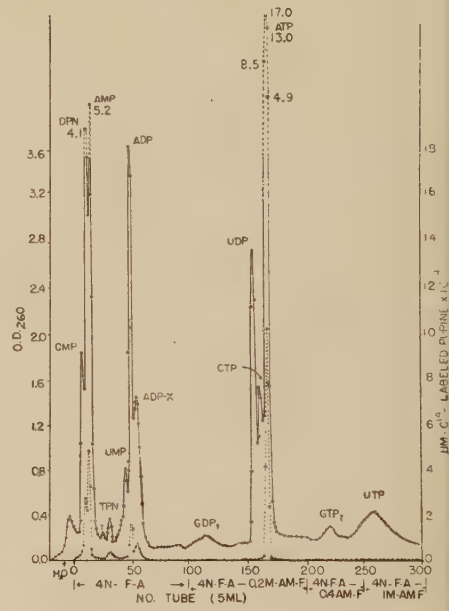


Fig. 1 (left). Ultraviolet absorption spectra of original *EE* and *EE* after dialysis.
Fig. 2 (right). Ultraviolet absorption spectra of fractions derived from *EE*.
Fig. 3 (upper left). Changes in DNA content during cultivation of heart tissue in different media. Equal proportions of the fractions were used in the composite media. When *HS*, *AS*, or *AI* were used alone, each was first diluted with an equal volume of Tyrode's solution.
Fig. 4 (lower left). Relative rates of incorporation of adenine-8-C^{14} into mixed nucleic acids of cultures, with different media. The labeled compound was employed at a 0.1 m*M* concentration in each case.
Fig. 5 (right). Chromatography of a perchloric acid extract of radioactive chick embryos (13 g). A 20 × 0.9 cm Dowex 1 column was used. Elutions were performed with formic acid and ammonium formate [6], and 5 ml fractions were collected. The open symbols are optical densities of the eluted fractions, while the solid circles are the corresponding radioactivities.

TABLE III

Incorporation of C^{14} of labeled fractions into nucleic acids of heart cultures. Equal proportions of the constituents were employed in the nutrient media.

Composition of medium	μM incorporated per gm of isolated material			
	Nucleo-protein	Mixed sodium nucleates	DNA	RNA
DEE + labeled *PS* (0.020 μM C^{14}-purine per ml)[1]	0.45	1.77	1.00	1.91
PS + labeled *DEE* (0.0105 μM C^{14}-purine per ml)[2]	5.255	1.17	0.64	1.34

[1] Contained in adenine nucleotides.
[2] Representing C^{14}-adenine and C^{14}-guanine in both DNA and RNA.

butions of both protein and non-protein components of *EE* to the synthesis of tissue protein is thus apparent. Table II indicates that RNA was utilized some 5 to 8 times as extensively as DNA. This is in accord with the known predominance of RNA in animal tissues (including embryonic heart). It is also noteworthy that about as much non-polynucleotide ribose as RNA ribose was taken from the medium (considering that RNA contains about 30 per cent of this pentose).

Since the degree of incorporation of isotopic adenine into tissue nucleic acids appears to be closely correlated with the rate of growth of fibroblasts [9], it was of interest to employ this criterion in studying the various nutrient fractions. The results in Fig. 4 are strikingly like those in Fig. 3 (in which DNA was measured). A combination of *DEE* and *HS* was fully equivalent (actually superior) to *EE*, with respect to the uptake of adenine-8-C^{14}; while *DEE* plus *PS*, or plus *AS*, was somewhat less effective at 6 days. Low rates of C^{14} incorporation (with virtually no growth) resulted with *HS*, *AS*, *AI*, or *DEE* alone, or with *DEE* plus *AI*.

Another approach was the use of labeled nucleotides and nucleic acids in studying the utilization of nutrient fractions (Table III). In the upper part of this table, the value of 0.45 for nucleoprotein represents the uptake of 2.5 per cent of the total C^{14} available in adenine nucleotides of the medium, during a 6-day cultivation period. The corresponding nucleoprotein value, 0.20, in the lower half of the table indicates a utilization of 2.0 per cent of the C^{14} in the nucleic acid purines. This approximately equal contribution of C^{14} by the two phases of the medium agrees with the finding (Table II)

that the nucleic acids of *EE* were utilized as extensively as the ribose nucleotides, for tissue nucleic acid synthesis.

The higher C^{14} content of RNA, as compared with DNA, was due to the much greater proportion of RNA in the tissue cultures. In point of fact, the *concentration* of C^{14} was considerably higher in DNA, as compared to RNA, in both experiments.

In examining the role of the nucleotide constituents of *EE*, it was of interest to subject these to chromatographic analysis. Because of the presence of interfering salts in the standard *EE* preparation, a direct perchloric acid extract of whole chick embryos was employed[1] (Fig. 5). The nucleotides of the embryos had been biologically labeled with adenine-8-C^{14} (10), so that radioactivity as well as nucleotide concentration could be measured in each chromatographic fraction. The nucleotide composition of each peak was ascertained from its ultraviolet absorption spectrum, and from E_{275}/E_{260} [6].

Ten acid-soluble nucleotides were identified in chick embryonic tissue: cytidine monophosphate, diphosphopyridine nucleotide, adenosine monophosphate, triphosphopyridine nucleotide, uridine monophosphate, adenosine diphosphate, uridine diphosphate, cytidine triphosphate, adenosine triphosphate, and uridine triphosphate. Guanosine di- and triphosphates are tentatively indicated in Fig. 5. Thymine compounds could not be detected. The substance designated ADP-X was an unidentified adenine compound.

ATP was by far the most abundant nucleotide, and contained the most C^{14}. Lesser amounts of C^{14} occurred in DPN, AMP, TPN, ADP, and ADP-X. Ninety-two per cent of the total C^{14} was recovered in these six peaks. The apparent absence of labeled guanine compounds contrasts with the C^{14} distribution observed in the nucleic acids of *EE*, in which guanine accounted for about half of the purine-C^{14} [10].

Apart from their multiple roles in enzymic processes, the nucleotides are generally believed to represent intermediate stages in the formation of polynucleotides. Hence it seems very likely that the nucleotides in *EE* were utilized directly for tissue nucleic acid synthesis.

Obviously the *EE* contains a great variety of substances, in addition to protein and nucleic acid constituents, so that the elucidation of the complete nutritional requirements of living cells *in vitro* represents a difficult task. However the growth-promoting properties of the *PS*, and particularly the *HS* preparation in the present paper, suggest that further fractionation studies may be helpful.

[1] This method, rather than heat coagulation, was used because it resulted in complete removal of nucleic acids.

SUMMARY

Fractionation of chick embryonic extract has demonstrated that both the diffusible substances and the high molecular constituents were required for the *in vitro* growth of heart fibroblasts. Growth was evaluated by increase in tissue DNA content, and by incorporation of C^{14}-labeled adenine. It was found that either the perchloric acid-soluble, or the heat-stable phase of embryonic extract could substitute fairly well for the components removed by dialysis.

Direct chemical analysis of the nutrient medium during cultivation revealed the extensive uptake by tissue cells of nucleotides and nucleic acids, as well as protein components. Also the utilization of C^{14}-labeled nucleotides and nucleic acids was demonstrated.

Analysis of nucleic acid-free chick embryonic extract by Dowex column chromatography revealed a variety of different nucleotides, of which ten were identified. These compounds appear to have a dual role: in enzymic systems, and as building blocks of polynucleotide molecules.

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EXPERIMENTAL PRODUCTION OF FILAMENTS AND NETWORKS IN CYTOPLASM OF *CICHORIEAE*

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The cytoplasmic components of *Lactuca sativa L.* were identified in a recent study [30], and methods were found which would permit the observation for many days of the living explanted cells. Methodological procedure and details of identifications are fully discussed in this paper and need not be reiterated here. It suffices to indicate that the epidermal and subepidermal cells from the midrib of a leaf of iceberg lettuce have distinct mitochondria, plastids, and spherosomes; and, in addition, possess heavy colloidal substances in their vacuoles, which under the action of certain basic dyes form copious precipitates. Of particular interest, however, is a tendency of these cells to form, after experimental treatment, peculiar polymorphic filamentous structures inside the cytoplasm. Similar structures were observed and described by Buvat [4, 5, 6, 7, 8, 9, 10] in portions of the root of *Cichorium intybus L.* sterilized, washed, and kept in water for a prolonged time. He states that the chondriome of the parenchyma cells of such material assumes very peculiar forms, showing long heterogeneous filaments which may reach dimensions of 100–150 μ in length, and which may anastomose to form vacuoles as large or even larger than the size of the nucleus. These structures, observed by him both in living material, and especially in preparations fixed by Regaud's method, occur in the cells along with some mitochondria and small rod-shaped forms which remain intact. In his explants made from such tissue, the long branching forms resolved into a normal chondriome. Water was considered responsible for these occurrences by effecting changes in the surface tension of the chondriosomes [5, 9, 10], and causing the individual chondriosomes to join end-to-end into long complex structures.

Dangeard and de Puytorac [13] and de Puytorac [25] studied the effects of tap and distilled water on the chondriome of parenchyma cells of root of *Cichorium intybus L.*, *Daucus carota L.*, and that of epidermal cells of

Allium cepa L. The formation of long irregularly branching chondriocontes, similar to those described by Buvat, was recorded only in *Cichorium intybus L.*

I have found that the peculiar long branching forms of cytoplasmic particulates can be induced to appear in epidermal and subepidermal cells of a number of plants closely related taxonomically, belonging to the tribe *Cichorieae: Lactuca sativa L., Cichorium endivia L., Cichorium intybus L., Cichorium intybus* cult. var., and *Taraxacum officinale* L. Common features of these laticiferous plants include similarities in their cytoplasmic particulate morphology and the presence of A-type vacuoles in the epidermal and subepidermal cells.

The purposes of this investigation were to determine the nature of the heterogeneous filaments and the conditions under which they appear in the cells. Because the results obtained for different plants were similar to those of *Lactuca sativa L.*, the presentation of material in this paper is restricted to that plant only.

EXPERIMENTS WITH *LACTUCA SATIVA L.*

Only those living cells which showed more or less definite cyclosis were investigated. Small incisions were made with a razor blade on the midrib of the leaves of iceberg lettuce. The small pieces of midrib were then lifted by forceps and floated upon 10 per cent sucrose solution in double distilled water. The tissues thus isolated contained two layers of cells, epidermal and subepidermal. Both layers were investigated by mounting the pieces for microscopic examination with the cutin side either up or down, which procedure made possible determination of the distribution of various cytoplasmic components throughout both layers.

When etiolated material which has previously been kept in the humidifier of a refrigerator (4°C) is mounted on the slide and examined by phase contrast optics, the cytoplasmic particulate appears to consist of spherical and rod-shaped bodies, with a number of larger round leucoplasts, often found in the vicinity of the nucleus (Fig. 1). Cyclosis of such cells is slow, admitting the possibility of taking photomicrographs. In material which shows light green coloring in the leaves, cyclosis is much faster; and the same cytoplasmic components can be seen, except that the leucoplasts now begin to show green color. When similar material is stained with Janus green B, only very few of the small spherical and granular forms become colored. Only these are considered as mitochondria [30]. The spherical

forms which do not stain at this stage with Janus green B are the spherosomes [29, 30].

Spherosomes are highly refractive, move faster than any other component in the cytoplasmic streams, and, when examined under oil immersion with phase contrast, are visible as luminous square bodies with rounded corners, which rotate about their axes and appear jet black after rotation. As a result of their differing refractive indices they are black and exhibit a halo in the photomicrographs under lower power (Figs. 2, 3, 6). The majority of the rod-shaped forms seen in the vicinity of the nucleus (Fig. 2) also do not stain with Janus green B. Similar bodies are also found in the parietal layers of the cytoplasm and in the trabeculae which traverse the central vacuole. They may be very variable in size and shape, and produce chlorophyll when head lettuce material is kept in a moist chamber in light for 10 to 20 days. Most of the rod-shaped forms, therefore, are considered here as colorless plastids [28, 30].

When material is mounted on a slide in sucrose solution and a cover slip is applied, the following changes can be observed after 3 to 4 hours: the water evaporating from the edge of the square cover slip causes the sugar solution to become more concentrated and seals off the preparation from access to air. The small piece of material in the center of the slide becomes surrounded by an air space directly adjacent to it and by a tight area of sucrose solution, creating relatively anaerobic conditions inside the preparation. Under these circumstances cells may remain alive for several days, and can be frequently examined and photographed under the microscope.

Photomicrographs were made with a 35 mm camera attachment (R. Winkel) using Zeiss Opton microscope with dark contrast phase optics, neofluar objective Ph 40/0.75, K oculars 8 × with magnification changer for factors 1.6 × and 2.5 ×, and green filter. Negatives were enlarged as specified.

Figs. 1–6. Living epidermal cells from midrib of a leaf of head lettuce.

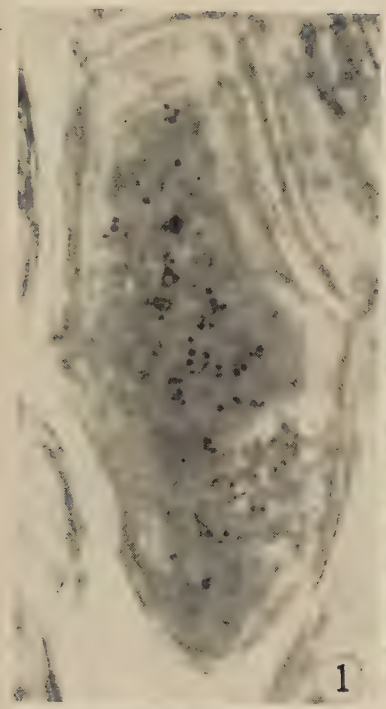
Fig. 1. Cell from an etiolated region mounted on the slide (cutin side down) in 10 per cent sucrose solution in double distilled water. Nearly spherical mitochondria and spherosomes are visible in the cytoplasm. In the vicinity of the nucleus round leucoplasts and rod-shaped colorless plastids are visible. × 923.

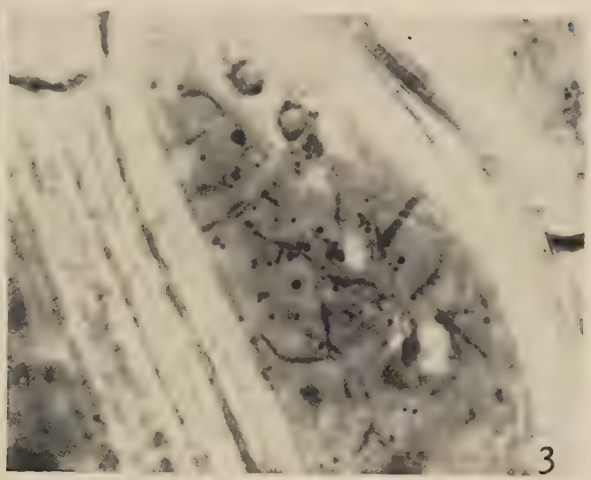
Fig. 2. Cytoplasmic particulates found in the vicinity of a nucleus in a cell stained with Janus green B. Round leucoplasts, rod-shaped colorless plastids and spherosomes (forms with halo) did not stain. Only few mitochondria showed blue color. × 940.

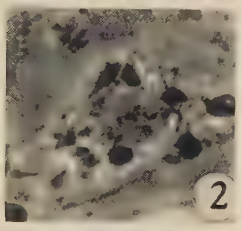
Fig. 3. Cell stained first with Janus green B, later mounted on the slide and kept for 3 hours under anaerobic condition. Branching filaments do not accumulate the dye. Spherical forms abundant in cytoplasm. White bodies — assimilation product. × 971.

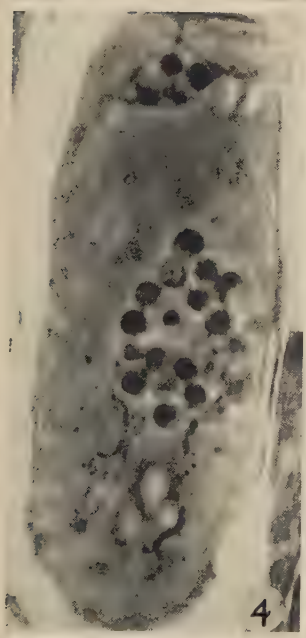
Figs. 4–5. Same cell photographed a few seconds apart. Filamentous structures change shape constantly, form loops, anastomoses. × 923.

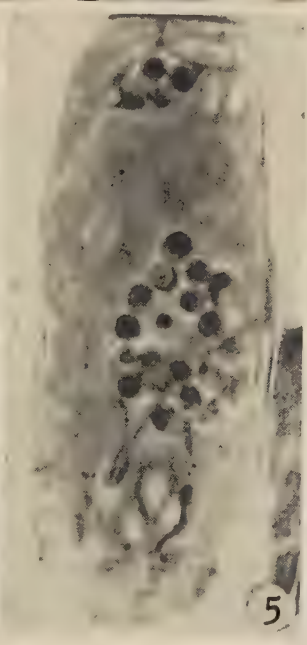
Fig. 6. Cell under anaerobic conditions for over 3 hours. Long filaments containing leucoplasts, form loops, and show end-to-end arrangements. Spherical forms free. × 1133.











Notable changes produced in the cells kept under anaerobic conditions for 3 or more hours are reflected as a gradual increase in viscosity, demonstrated by slower motion of the particulate and thicker strands of the cytoplasm. The colorless rod-shaped plastids become arranged end-to-end and often move as a unit, exhibiting constricted connections. Later, the complex structure exhibits a sheath or membrane which can be either an interface layer of cytoplasm, or an extension of the membrane of the plastids [12]. Individual units of the complex structures can be distinguished at first (in some forms of Fig. 6), but eventually the distinction disappears and the particulate assumes the forms of long branching filaments (Figs. 3, 6). These are in continuous motion, branching and anastomosing, to form loops and vacuoles. They may envelop leucoplasts which are found free in the cytoplasm. To show how fast the structures change their shape, the same cell is shown photographed at the same focus level after a short interval to change the film (Figs. 5, 4), about 5 seconds. The dark body to the right at the bottom of the cell (Figs. 5, 4) changes in thickness, while the bodies to the left form an anastomosing loop. The polymorphic bodies can assume still more bizarre forms appearing as thick lumps, which connect with the network of filaments (Fig. 7). This material was first stained with Janus green B and afterwards kept under anaerobic condition for 5 hours.

Head lettuce material which was used in experimental virescence and was accordingly kept in a moist chamber exposed to light for 10–20 days, showed plastids with considerable decreased viscosity. Leucoplasts and chloroplasts which were seen before in the shape of oval or almost round bodies (Figs. 2, 4, 5, 7), now became spindle-shaped and often exhibited attenuated protrusions. When such material was kept for a number of hours under anaerobic conditions, the small rod-shaped and granular plastids, which now showed green color, became involved in the formation of fila-

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Figs. 7–10. Living epidermal cells of lettuce. Fig. 11. Cell killed by 30 per cent acetic acid.
Fig. 7. Cell stained with Janus green B and kept on the slide for 5 hours. Filamentous structures assumed the appearance of thick polymorphic masses, which change shape continuously. × 923.
Fig. 8. Cell from material under experimental virescence. Plastid material less viscous. Ten per cent sucrose, for 3–4 hours under anaerobic conditions. × 733.
Fig. 9. Virescence of small former colorless plastids. Under anaerobic condition for 4 hours. Small green plastids become involved in the formation of networks together with attenuated protrusions of chloroplasts. × 733.
Fig. 10. Material mounted directly in liquid petrolatum. After 4½ hours the heterogeneous filaments made their appearance. × 1020.
Fig. 11. Cell which contained filamentous networks was killed by 30 per cent acetic acid. Filamentous structures remained intact. × 1020.

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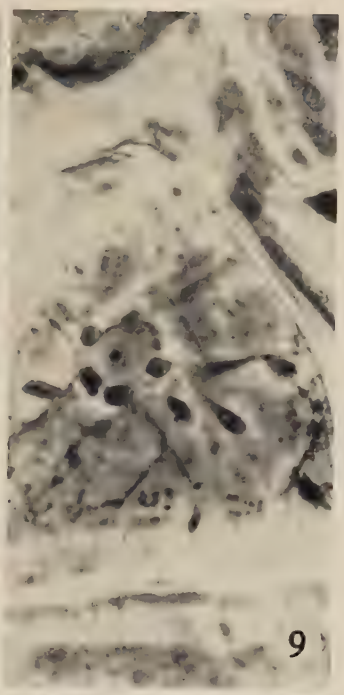
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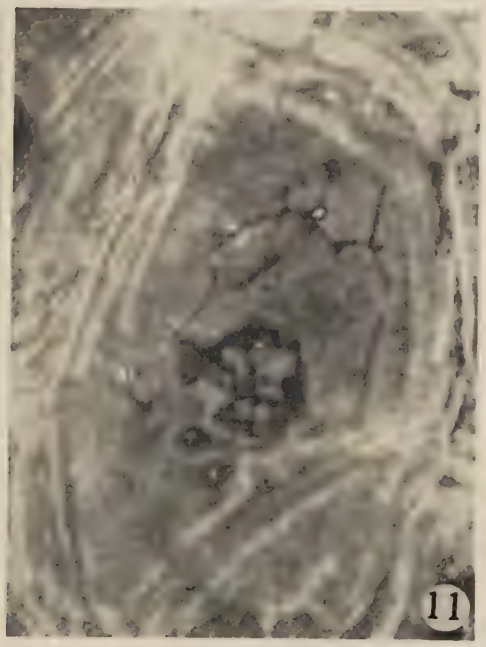
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ments and networks, together with the attenuated protrusions of the chloroplasts (Fig. 9). In another cell elasticity of plastid material under these conditions is shown in Fig. 8.

Significantly, in all the preparations observed spherosomes never took part in the formation of the heterogeneous filaments, and are plainly visible in all the photomicrographs as black bodies surrounded by a lighter halo. The oval bodies of ergastic material (possibly inulin), which appear white under phase contrast (Figs. 2, 3, 4, 5), also do not participate in the formation of the networks.

When the preparation, containing a well-developed net of filaments (Figs. 3, 6) is opened to air and perfused with a new supply of sugar solution, the long filaments break down into individual units, the cyclosis resumes its previous speed, and the preparations appear just as when freshly mounted. If the material has not already been stained, it can be stained at this time either by Janus green B or by combined staining with Janus green B and Neutral red [30]. The advantage of this latter stain consists in coloring red the precipitate of A-type vacuoles [1, 2, 3], and thus separating it from blue colored mitochondria.

After the cytoplasmic particulate resumes its original appearance of small spheres, granules and rod-shaped forms, the experimental formation of long filaments can be repeated again in the manner described above. In a few hours the long heterogeneous filaments again make their appearance.

It is possible to observe the formation of filamentous networks under anaerobic conditions in material previously stained either with Neutral red, or with the combination stain of Janus green B and Neutral red, and in which the heavy colloidal substances of the vacuoles had precipitated. That there is no connection between the filamentous networks and the colloidal substances of the vacuoles is thus ascertained.

The breaking down of these filaments into normal looking particulate can be achieved even without lifting the cover slip. It is sufficient to drop fresh sugar solution along the rim, and to draw it under the cover slip by means of filter paper placed at the opposite side. The whole procedure can be observed under the microscope.

Mounting of material in oil. -The use of medicinal liquid petrolatum (White Russian oil) as a control over sudden osmotic changes that might be induced in vacuoles by aqueous media was made by Bailey [1, 2]. He found also that the oil is a very good medium for keeping the cambium initials alive and actively streaming for 500 hours. In my experiments liquid petrolatum and olive oil were used for mounting the tissue being

studied for effects on cytoplasmic particulates of the absence of both water and oxygen.

In the first series of experiments pieces of fresh material were mounted on a slide under a cover glass directly in liquid petrolatum or in olive oil. In about 4–5 hours the typical filaments and networks made their appearance in both mounting media. In Fig. 10 such a cell is represented. The network is well outlined, and as usual the spherosomes are not connected with it. The nucleus again is visible with the ring of leucoplasts about its surface. As the oil medium does not make a good phase contrast, the photomicrograph is somewhat dark.

If pieces of epidermis are floated, or suspended in olive oil in a shallow open dish for a period of time even exceeding 24 hours, the filamentous structures fail to appear. Apparently sufficient air diffuses into the medium to prevent the formation of anaerobic conditions.

Action of acetic acid.—To determine the reaction of filamentous structures towards acetic acid, material which contains networks and filaments is subjected to 30 per cent glacial acetic acid. The acid is dropped from the side over the cover slip and is drawn under it by means of filter paper located at the opposite side, while changes in the cell are recorded through the microscope. At the moment acid reaches the cell a shudder passes through the cytoplasm and the filamentous structures thicken slightly and become still, but they do not dissolve in this strong acid. In Fig. 11 a cell is photographed half an hour after the acid has reached it. The nucleus has shriveled, the spherosomes are not distinct, but the network of filaments is clearly recognizable.

When fresh material is stained beforehand with Janus green B, and 30 per cent glacial acetic acid is dropped from the side of the cover slip during observation of the preparation under the microscope, the blue colored mitochondria become vesiculate and dissolve after the acid reaches the cell. The leucoplasts and the small rod-shaped and filamentous plastids, which do not take Janus green B dye, do not dissolve when acid reaches the cell. They are still recognizable in the preparations 4 days after the material is killed by acetic acid.

DISCUSSION

Because the recognition and identification of cytoplasmic components are essential for the study of localization of enzyme systems in the cells, it becomes extremely important from the theoretical point of view to ascertain

whether there are one, two, or more categories of granules in the cytoplasm of plant cells [31].

This question has caused considerable controversy among plant cytologists in the past, and unanimity has not been reached up to the present time. For adequate reviews see Newcomer [21, 22] and Sorokin [29]. The proponents of one category of granules base their conclusions on fixed material. The usual mitochondrial methods, and those specially adapted for plant cells [32, 23, 20] do not differentiate between various cytoplasmic particulates. By using living and fixed material concomitantly Küster [19], Perner [24], and Drawert [14] have identified three permanent components of the cytoplasm of plant cells: mitochondria, plastids, and microsomes (spherosomes). Dangeard [12], recognizing the constant presence of microsomes in the cytoplasm of living plant cells, regards their exact nature as uncertain. However, he admits a clear distinction between chondriosomes (mitochondria) and leucoplasts. The latter, accordingly, may have either the shape of the chloroplasts, or that of a ribbon or filament. A term *mitoplastes* is reserved for such plastids, which resemble chondriosomes in their appearance, but are distinguished from them by their higher plasticity, which is manifest in constant changes of shape and frequent branching. In his later works Guilliermond [16] also accepts three categories of granules in the cytoplasm of plant cells: the lipid granules, and two types of chondriosomes. The first category is considered to be permanent ergastic substance, whereas active and inactive chondriosomes exhibit in phanerogams the same shape, and, until they develop pigment, cannot be told apart in the meristems and, usually, in mature cells.

Studies of my material indicate that in epidermal and subepidermal cells there are always three distinct types of bodies in the cytoplasm: mitochondria, plastids, and spherosomes. Their identification is effected on the basis of different staining reaction with Janus green B and different properties exhibited under phase contrast microscopy [28, 29, 30].

Buvat [7] accepts the idea that the cytoplasmic particulate of the cells of *Cichorium intybus L.* is heterogeneous, and separates its units on the basis of their reaction towards water. Accordingly, leucoplasts are considered bodies which transform under influence of water into voluminous vesicles, whereas under the same condition, one type of chondriosome undergoes hypertrophy (but rarely vesiculates), and the other type does not react in any way. As no other identification of elements is given, I presumed that the particles of Buvat's second category participate in the formation of the long branching filaments under discussion and correspond to the active

chondriosomes of Guilliermond [16], or to plastids, in my terminology. However, the question remains confused, as the long filaments upon breaking down are interpreted by Buvat [10] as mitochondria. The chondriosomes which do not react in any way towards water must correspond to the inactive group of Guilliermond [16], or to mitochondria and spherosomes of the present study.

The heterogeneous filaments of Buvat are in their turn built up of units which either are just associated end-to-end, or form anastomoses. The first type dissociates readily with resumption of cyclosis, whereas the second type requires a longer time for this process [7]. As to the immediate cause of the formation of long filamentous structures, Buvat [10] regards that the stopping of cytoplasmic streaming (due to submersion) enables the chondriosomes to take up water, and to join together. Water supposedly modifies the bond between the macromolecules in cytoplasm. Reversibly, the breaking down of these long structures is effected by increasing the velocity of cytoplasmic streaming, which can be achieved by placing material containing these structures under the light microscope.

As has been indicated in present experiments the polymorphic structures occurred in epidermal and subepidermal cells of *Lactuca sativa L.* after material was kept under anaerobic conditions for a period of over 3 hours. As soon as air is admitted to the preparations, the structures break down into the original components of the cell. In experiments by Buvat [6] the heterogeneous filaments failed to appear when living sectioned material was mounted on the slide in such a way as to provide free access of air between the sections, and when it was regularly perfused with fresh water. The cell remained alive for two weeks and was regularly observed under the microscope. However, when he mounted sections in distilled water, and put them aside for several days without submitting them to light of the microscope, and without changing the water in which they were mounted, then the heterogeneous filaments were present. Similarly, long filaments were seen in sections from blocks of living roots kept under water, provided the examination was made immediately after mounting [7]. All this suggests that the principal factor, the appearance of filamentous structures in the cytoplasm of the cells mentioned, is the anaerobic state of the mounting media.

Finally, our experiments with mounting the material in mineral and olive oils which still yielded the filamentous structures after an interval from 4 to 5 hours, disproves conclusively the theory of water being responsible for the occurrence of long branching filaments in the cytoplasm of the cells mentioned. By the assumption that an anaerobic state of the medium is

conducive to their appearance, all cases of presence and absence of these structures in the experiments conducted by Buvat could be explained.

Because mitochondria are widely recognized to be extremely sensitive to acetic acid, all classical methods for mitochondrial fixation in both plant and animal cells are based on exclusion of acetic acid from the fixing fluid. According to Dangeard [11], Dangeard and de Puytorac [13] and de Puytorac [25] acetic acid even in dilute concentrations caused mitochondria to swell, form hollow spheres, and finally to become completely vesiculate. Rezende-Pinto [26] finds that plastids are not affected by acetic acid-containing fixatives and separates them from mitochondria on this basis. In the present study a strong concentration of acetic acid is shown to preserve the filamentous structures under discussion, and therefore eliminates the possibility of considering them as being composed of mitochondria joined end-to-end. The negative reaction of these structures towards Janus green B adds another reason for excluding them from the category of mitochondria.

The experiments with double staining of preparations by Janus green B and Neutral red show that the heavy colloidal substances of the vacuoles have no connection with the formation of the polymorphic structures under discussion, as this method permits one to see in the same preparation the vacuolar precipitate as a red deposit, the mitochondria with a blue color, and the polymorphic filaments or networks as independent structures in the cytoplasm. The spherosomes also do not participate in the formation of these heterogeneous structures. Therefore, it is logical to conclude that the colorless plastids compose the main bulk of the filaments thus formed.

The plastids are known to be sensitive towards environmental conditions. As observed by Sorokin [27], they round up in hypertonic solutions and stretch out in hypotonic media. They frequently exhibit changes in viscosity which are manifest in amoeboid movement and polymorphism, numerous observations of which are cited by Guilliermond *et al.* [17]. Küster [18] records their ability to stretch and form filamentous appendices, and Dangeard [12] has demonstrated experimentally that these protrusions arise as irregular elongations of the plastid's membrane. However, the particular forms which the filamentous structures under discussion assume have been observed only in plants of the tribe *Cichoriceae*, which are laticiferous. Although the question of the origin of latex is not sufficiently investigated, it is supposed that latex particles are formed either in the cytoplasm, or in the plastids of the laticifers [17, 15]. It is not implied here that the filamentous structures are latex, but it raises the question whether plastids, which may have the potentiality of producing latex in the laticifers, may react under

anaerobic conditions by changing their viscosity, joining end-to-end, anastomosing and forming filaments and networks.

SUMMARY

1. The cytoplasmic particulate of epidermal cells of *Lactuca sativa L.* and of a number of plants belonging to the tribe *Cichorieae* consists of mitochondria, plastids and spherosomes. Their identification is effected on the bases of differential staining with Janus green B, different properties exhibited under the phase contrast microscope, and by experimental virescence of colorless plastids.

2. When living epidermal cells of *Cichorieae* are mounted on a slide under anaerobic conditions for more than 3 hours, long filamentous structures appear in the cytoplasm. They branch, anastomose, form loops, networks, and change shape constantly.

3. Spherical elements of the cytoplasm do not participate in the formation of branching filaments.

4. Filaments and networks may also be produced in cells mounted under anaerobic conditions in liquid petrolatum and in olive oil.

5. The filaments and networks are not dissolved in 30 per cent acetic acid.

6. The heterogeneous filaments formed under anaerobic conditions are considered to be composed of modified plastids joined end-to-end and enveloped by a membrane which could be either an extension of the plastid membrane, or an interface membrane between the plastids and the cytoplasm.

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Dr. I. W. Bailey has given me the benefit of his criticism of this paper.

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SYNTHESIS OF PROTEIN AND RIBONUCLEIC ACID IN RAT LIVER DURING REFEEDING AFTER STARVATION[1]

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A NUMBER of authors [1, 19, 24, 29] have shown that starvation results in rapid loss of protein and of ribonucleic acid from the liver, in excess of the corresponding losses throughout the rest of the body. Enzyme studies have shown that this is not simply a loss of storage material, but is actually a loss of functional liver tissue [24, 29]. These losses have been shown to occur without significant change in the total cell number or in the total desoxyribonucleic acid content of the liver [19, 23], with a possible exception in the case of very young animals [12, 17]. Lagerstedt has made a detailed cytological study of rat liver during starvation and refeeding [20], but the rapid synthesis of protein and ribonucleic acid which occurs during recovery from starvation has not been studied extensively by biochemical methods.

The process of recovery from starvation thus involves rapid production of functional liver constituents without the complication of cell division, and the present investigation was undertaken because it seemed probable that a study of protein and nucleic acid metabolism in the various cell fractions during this recovery period would yield information concerning the role played by the various intracellular structures in the synthesis of protein and ribonucleic acid during non-mitotic growth.

MATERIALS AND METHODS

In all the experiments to be described, groups of male albino rats were deprived of food for six days, and then they were given access to food *ad libitum*; they were allowed free access to water throughout each experiment. The diet consisted of "Friskies" dog pellets in the first experiment, and of Purina dog chow in the later experiments; the food was ground to facilitate weighing for the food consumption records. At appropriate times the animals were killed with ether in groups of four;

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three hours before sacrifice, each rat received glycine-2-C^{14} and inorganic phosphate-P^{32} in a single intraperitoneal injection.[1] The livers were removed, weighed, perfused with isotonic sucrose solution, and pooled for fractionation by differential centrifugation into a nuclear, a large mitochondrial, a small mitochondrial, a microsomal, and a supernatant fluid fraction [21]. The two mitochondrial sub-fractions were separated by the simplified procedure described previously [22]. Each fraction and the unfractionated homogenates were analyzed for nucleic acids by Schneider's method [28] and for protein-nitrogen by a nesslerization procedure [33]. Nuclei were counted in the unfractionated homogenate by a technic described previously [21]. The analytical results were calculated as amounts in the average cell and in the average whole liver.

The procedure for the isolation of protein and nucleic acid samples for determination of specific activities was based on the method described by Barnum and Huseby [3]. Each tissue sample was mixed with an equal volume of 20 per cent NaCl solution and heated for one hour in a boiling water bath. The protein precipitate was removed by filtration, extracted with 5 per cent trichloracetic acid at 80°C and then with conventional lipid solvents, and dried. The nucleic acids were precipitated from the chilled filtrate by the addition of 2 volumes of ethyl alcohol. The nucleic acid precipitate was dissolved in water and reprecipitated by the addition of an equal volume of cold 10 per cent trichloracetic acid. After centrifugation, the nucleic acid precipitate was washed twice with 5 per cent trichloracetic acid and was then suspended in 0.1 *N* NaOH and heated at 80°C for 20 minutes. Any trace of precipitate remaining was discarded and the desoxyribosenucleic acid (DNA) was precipitated by making the filtrate 0.1 *N* in HCl; the ribose nucleic acid (RNA) remained in the supernate. The DNA precipitate was extracted again with 0.1 *N* NaOH for 10 minutes and reprecipitated as before. The DNA was brought into solution by extracting the precipitate with 5 per cent trichloracetic acid at 80°C for 20 minutes; any trace of solid remaining was discarded.

The P^{32} radioactivity of the nucleic acid extracts was measured in an annulus counter[2] which registered only P^{32} since its glass walls were thick enough to absorb all of the C^{14} radioactivity. For measurement of the C^{14} activity of these solutions, aliquots were subjected to wet combustion (Van Slyke-Folch); the $C^{14}O_2$ thus separated from the P^{32} was trapped in NaOH and converted to $BaCO_3$. The protein samples were counted under an end-window counter, with and without an aluminum disc which absorbed all of the C^{14} radioactivity and reduced the P^{32} count to 73 per cent of that obtained without the absorber; the data so obtained were used to calculate the C^{14} radioactivity of the protein samples. The specific activity of the uncombined glycine was determined by the procedure described previously [2], except that the dimedon precipitate was dissolved in dilute alkali and reprecipitated with 2–3 drops of glacial acetic acid. The specific activity of the acid-soluble phosphorus was determined by measuring the P^{32} radioactivity and total phosphorus content of a trichloracetic acid extract prepared from each homogenate.

[1] The glycine-2-C^{14} was obtained from Tracerlab, Inc. on allocation from the U.S. Atomic Energy Commission. The orthophosphate-P^{32} was supplied by Dr. E. C. Albright of the Department of Medicine, University of Wisconsin, on allocation from the U.S. Atomic Energy Commission.

[2] Obtained from Radiation Counter Laboratories, Skokie, Ill.

The specific activity measurements were made on the nucleic acids not only from nuclear fractions obtained in the sucrose medium ("sucrose nuclei"), but also from "acid-washed nuclei" prepared by washing "sucrose nuclei" first with 2 per cent citric acid and then repeatedly with 2 per cent acetic acid. This extraction removes substantially all of the cytoplasmic contamination, but also removes some nuclear protein and RNA [15].

For microscopic examination, a small piece of each liver was fixed in Carnoy's solution, and sections were stained with hemalum and eosin or by the Feulgen reaction. Nuclear diameters were measured in arbitrary units with an eyepiece micrometer and an oil immersion lens, in relatively thick sections (10 microns); only those nuclei were measured which were entirely within the section, as determined by careful focussing. The diameter was measured on about 100 nuclei from each liver, and about 250 nuclei from each liver were classified with reference to the surface morphology.

In the first experiment, the average initial weight of the animals was 250 g. The glycine-2-C^{14} dosage was 17 μc (1 mg) per rat and the inorganic phosphate-P^{32} dosage was 200 μc per kg body weight. Animals were sacrificed and the livers fractionated after 6 days of starvation, and after 1, 2, 4, 7, and 12 days of refeeding. An initial control group was studied at the time starvation was begun.

In the second experiment, the average initial weight of the animals was 200 g; the glycine-2-C^{14} dosage was 67 μc (4 mg) per kg body weight, and inorganic phosphate-P^{32} dosage was 200 μc per kg body weight. Animals were sacrificed and the livers fractionated after 6 days of starvation, and after 1, 2, 4, and 7 days of refeeding.

In the third experiment, the average initial weight of the animals was 180 g. The inorganic phosphate-P^{23} dosage was 20 μc per kg body weight and the glycine-2-C^{14} dosage was the same as in the previous experiment. Animals were sacrificed after 6 days of starvation and after 6, 12, 18, 24, 30, and 48 hours of refeeding. In this experiment the livers were not fractionated.

In the fourth experiment, the average initial weight of the rats was 267 g. No radioactive labeling was employed. The nuclei were isolated from homogenates prepared in 2 per cent citric acid; they were washed three times in 1 per cent citric acid and were analyzed as in the previous experiments.

RESULTS

The liver and cell weights, and the total number of cells in the average liver for Experiments 1 and 2 are given in Table I. The total number of cells in the average liver did not vary significantly from normal during 6 days' starvation or during subsequent refeeding. The marked changes in the weight of the average cell, and in its content of ribonucleic acid (RNA) and protein-nitrogen, are therefore proportional to the corresponding changes in the average whole liver. After 6 days' starvation the weight of the average liver and of the average hepatic cell was reduced to about one-half the normal value. On refeeding, rapid increases in the liver and liver cell weights occurred, reaching the normal values by about the 7th day; the most rapid increase in weight occurred during the first day of refeeding.

TABLE I

Liver and cell weights and total number of liver cells.

Experiments 1 and 2.

Experiment	Liver weights grams		Cell weights $g \times 10^{-9}$		Total no. cells $\times 10^9$	
	1	2	1	2	1	2
Control	14.0	—	7.15	—	1.96	—
6 d. Starv.	6.7	6.9	3.72	3.07	1.81	2.25
1 d. Refeed.	9.3	10.5	5.16	4.68	1.81	2.25
2 d. „	10.5	11.1	5.75	6.10	1.82	1.82
4 d. „	13.2	11.0	6.42	5.62	2.04	1.95
7 d. „	14.1	13.1	7.64	5.76	1.85	2.28
12 d. „	16.1	—	7.25	—	2.22	—

Analytical Results

Experiments 1 and 2.—Fig. 1, A and D, gives the changes in protein-nitrogen content of the average liver and of each cell fraction of the average liver for Experiments 1 and 2; the corresponding changes in RNA content are given in Fig. 2, A and D. During 6 days of starvation the total protein and total RNA content of the average liver dropped to about one-half the normal values; these losses corresponded closely to the losses in liver and cell weight noted above. During refeeding, the total liver protein and RNA increased in more or less parallel fashion, and they approached normal values after approximately 7 days. In the various cell fractions, the protein and RNA decreased nearly proportionally during starvation, except that the decrease was relatively smaller in the nuclear fraction and disproportionally large in the RNA-containing small mitochondria. During the first day of refeeding, both the protein and the RNA of the small mitochondria increased more extensively than did any other cell fraction. After the first day of refeeding, the RNA and protein of all the cytoplasmic fractions increased in a more or less linear fashion. In both experiments there was a well-marked delay in the increase in total cytoplasmic protein and RNA during the first 24 hours (Fig. 3 and 4); the increase in total liver protein and RNA also did not begin until about 24 hours after the resumption of feeding during the first experiment, but in the second experiment this delay was masked by the early rise in nuclear protein (see below).

The nuclear fraction behaved differently from any of the cytoplasmic fractions (see especially Figs. 3 and 4). In the first experiment, the protein-nitrogen content of this fraction showed a sharp maximum on the fourth day of refeeding; in the second experiment it showed a peak on the first day, and it was again elevated, though to a lesser extent, on the 7th day. In contrast, the total cytoplasmic protein in both experiments increased in an almost linear fashion, except for a single sharp increase which corresponded in time and magnitude to the loss of protein from the nuclear fraction (designated "*x*" in Fig. 3). The net cytoplasmic protein (total minus the

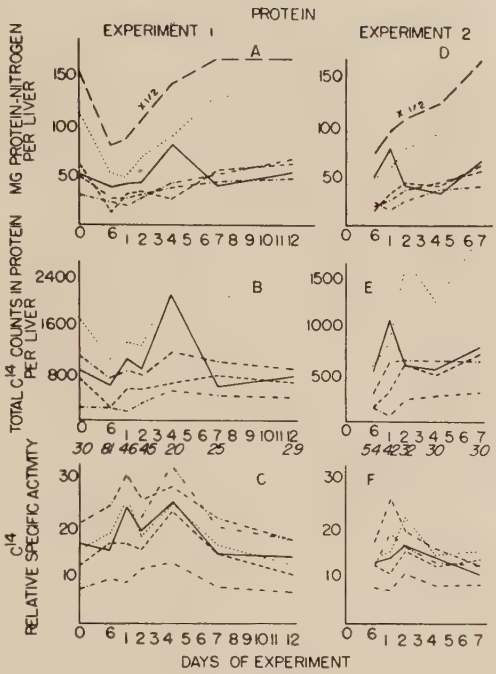


Fig. 1. Changes in quantity and radioactive labeling of protein during Experiments 1 and 2. The curves for the various cell fractions are identified as follows: Total protein of liver — — —; nuclear fraction, from sucrose homogenates ——; nuclear fraction from sucrose homogenates, washed in acid (see text) — · · — · · large mitochondria fraction — · — · ; small mitochondria fraction – – – –; microsome fraction · — — · ; supernatant fraction · · · · ·. The italicized numbers below graphs B and E are the values for the specific activity of the uncombined glycine-2-C^{14} in the liver, expressed as counts/min/μg glycine. Relative specific activity is expressed as counts/min/mg protein divided by the counts/min/μg uncombined glycine. Total counts per liver for each fraction is expressed as the relative specific activity of the protein times mg protein. Days 0 through 6 indicate the period of starvation; days 1 through 7 and 1 through 12 indicate the period of refeeding.

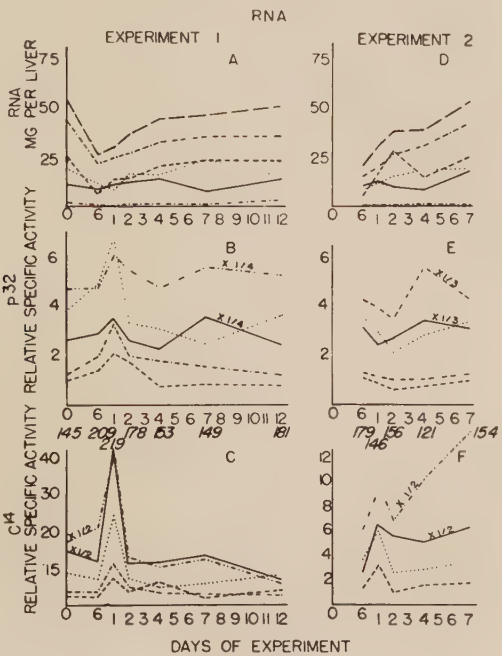


Fig. 2. Changes in quantity and radioactive labeling of RNA during Experiments 1 and 2. The curves for the various cell fractions are identified as in Fig. 1. The italicized numbers below graphs B and E are the values for the specific activity of the acid-soluble phosphate-P^{32} (ASP) expressed as counts/min/μg ASP. The relative specific activity for C^{14} is expressed as counts/min/mg RNA divided by counts/min/μg uncombined glycine; that for P^{32} is expressed as counts/min/mg RNA divided by counts/min/μg ASP.

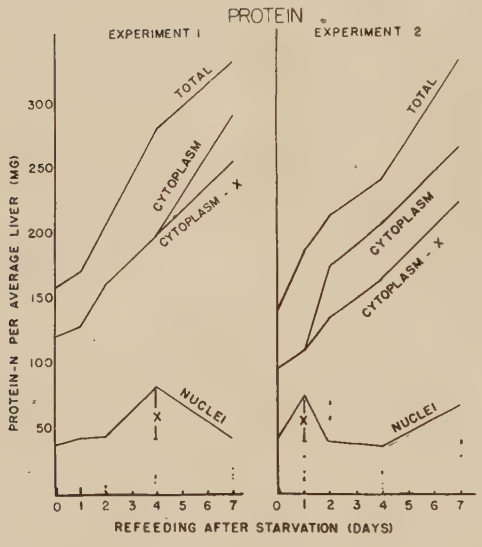


Fig. 3. Changes in the total liver protein, in the protein of the nuclear fraction, and in the total cytoplasmic protein, and in the net cytoplasmic protein (cytoplasm $-x$). The net cytoplasmic protein was calculated as the difference between the total cytoplasmic protein and "x", the decrease in protein content of the nuclear fraction after day 4 in Experiment 1 and after day 1 in Experiment 2. The dots at the bottom of the graphs give the per cent of wrinkled nuclei in the individual livers as determined by microscopic examination of each liver. In several cases the points for individual livers overlap.

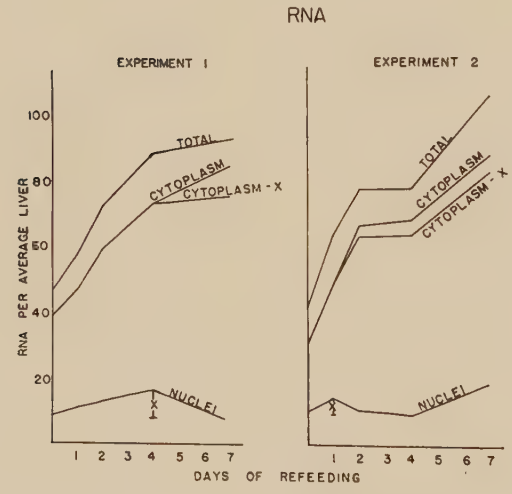


Fig. 4. Changes in RNA corresponding to the changes in protein shown in Fig. 3.

amount "x" lost by the nuclear fraction) increased in a straight line except for the moderate delay which occurred during the first day. It is noteworthy that the rate of increase of the total protein-nitrogen of the liver was greatest between the first and fourth days in the first experiment, and during the first day and again between the fourth and 7th days of refeeding in the second experiment, at times corresponding to the nuclear protein maxima.

The changes in nuclear RNA (Fig. 4) in both experiments paralleled the changes in nuclear protein, the peaks occurring at the same time as the protein peaks, but of

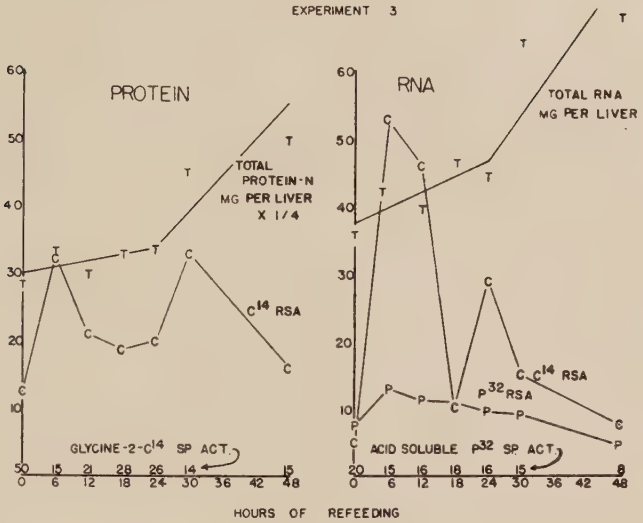


Fig. 5. Changes in the quantity and labeling of total protein and RNA of the liver during the first 48 hours of refeeding after starvation. RSA, the relative specific activities, are expressed as in Figs. 1 and 2.

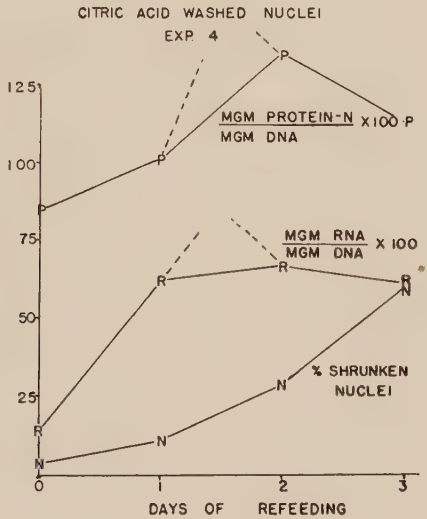


Fig. 6. Changes in the protein and RNA content per unit of DNA in citric acid-washed nuclei from livers during the first three days of refeeding after 6 days' starvation.

smaller relative magnitude. However, unlike the net cytoplasmic protein, which increased substantially in a straight line, the net cytoplasmic RNA (total minus the amount lost by the nuclear fraction) increased in stepwise fashion. After the fourth day in the first experiment, and between the second and fourth days in the second experiment, there was practically no increase in the total RNA of the liver or in the

net RNA of the cytoplasm. Thus the RNA increases occurred concurrently with the increases in nuclear protein.

Experiment 3. –The results of this short term experiment are given in Fig. 5. A well-marked delay of about 24 hours in the net synthesis of protein and of RNA was observed, similar to that occurring in the total liver protein in Experiment 1, and in the cytoplasmic protein of Experiments 1 and 2.

Experiment 4.— In this experiment, nuclei were prepared in citric acid in order to reduce to a minimum contamination of the nuclear fraction by cytoplasmic material. The results of this experiment are presented in Fig. 6. A peak in nuclear protein was observed this time also, but on the second day of refeeding. The increase in this experiment is smaller than those in the first two experiments, in which the protein of the nuclear fraction increased to twice the initial value. It is probable, in view of the increase in shrunken nuclei already apparent at this time (see below), that the real peak occurred between the first and second days as suggested by the dotted lines in Fig. 6. The nuclear RNA also reached a poorly defined maximum at the second day, and it is supposed that the maximum actually occurred between the first and second days, as suggested by the dotted lines.

Isotopic Labeling Results

Experiments 1 and 2. The values for the C^{14} specific activity of the uncombined glycine at sacrifice three hours after injection are indicated by the italicized numbers at the bottom of Fig. 1, B and E; they are expressed as c/m/μg glycine. The specific activity was high in the starved animals and it receded toward normal values as recovery from starvation progressed; in the starved animals of Experiment 1 it was about two times the values found in the normal control animals.

The P^{32} specific activity of the acid-soluble phosphate of the liver, expressed as c/m/μg acid soluble P, is indicated by the italicized numbers at the bottom of Fig. 2, B and E. As with the uncombined glycine, the specific activity of the acid soluble phosphate three hours after injection was high in the starved animals and receded toward normal values during recovery from starvation. The fluctuations in the specific activity of the acid-soluble phosphate during starvation and recovery were smaller than the corresponding fluctuations in the specific activity of the uncombined glycine.

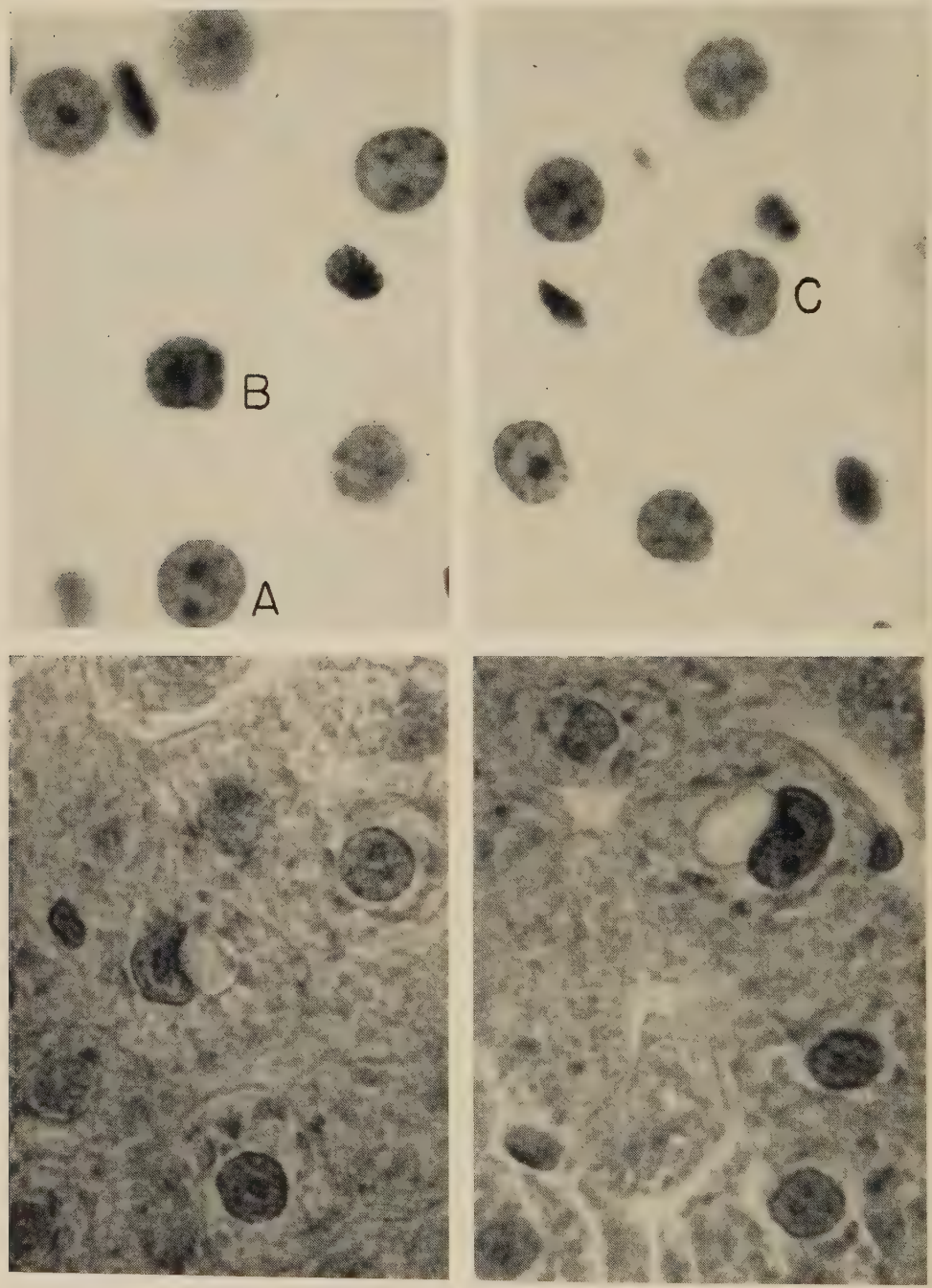
The C^{14} specific activity of the proteins is given in Fig. 1, C and F. The values given are relative values, expressed as measured C^{14} counts per minute per mg of protein divided by the counts per minute per μg of uncombined glycine. A measure of the total incorporation of C^{14} radioactivity into the protein of each cell fraction was obtained by multiplying the relative C^{14} specific activity of the protein in each fraction by the quantity of protein-nitrogen in that fraction of the average liver; these values are given in Fig. 1, B and E. In the case of the nuclear fraction, the calculation of total incorporation of radioactivity was based on the specific activity of the protein of the "sucrose nuclei". In general, the relative specific activity of the protein in each of the cell fractions tended to be high during the early phase of recovery from starvation, and receded toward normal values as the restoration of liver protein approached completion. The total incorporation, which probably gives

a better indication of protein metabolic activity, showed interesting differences among the cell fractions. The sudden increase in nuclear protein which occurred on the fourth day in the first experiment, and on the first day of refeeding in the second experiment, was accompanied by a corresponding abrupt peak in the total incorporation of C^{14} radioactivity into the nuclear protein. In the first experiment this peak in the total incorporation was accompanied by a peak in the specific activity of the protein of the "acid-washed nuclei" also. The sudden increase in nuclear protein was accompanied by or immediately followed by a large increase in both the 3-hour specific activity and the 3-hour total incorporation of C^{14} radioactivity into the protein of the supernatant fluid fraction. A less dramatic increase in the quantity and total labeling of the nuclear protein was also observed on the 7th day of the second experiment, although the specific activity of the nuclear protein was not increased. In this case, also, the total incorporation of C^{14} into the supernatant protein was large. In the cytoplasmic particle fractions, the total incorporation was low in starvation and it rose toward normal values during the first two four days of recovery, in contrast to the supernatant fluid fraction; thus the total incorporation into the protein of the cytoplasmic particles showed very little response to the relatively large fluctuations observed in the quantity and labeling of nuclear protein.

The C^{14} specific activity of the RNA is given in Fig. 2, C and F. As was the case with the protein, the values given are relative specific activities, expressed as counts per minute per mg of RNA divided by the counts per minute per μg of uncombined glycine. The RNA content of the large mitochondria fraction was too small to permit isolation of the RNA in sufficient quantity for radioactivity measurements. The C^{14} specific activity of the RNA in each cell fraction rose to a peak after one day of refeeding in both experiments, although the increases in the second experiment were much smaller than the corresponding changes in the first experiment. The relative specific activity of the RNA in each cell fraction dropped to normal or below normal values by the second day of refeeding and remained in this range throughout the recovery period.

The values for P^{32} relative specific activity of the RNA in each cell fraction, expressed as c/m/mg RNA divided by c/m/μg acid soluble P, are given in Fig. 2, B and E, and follow a course quite similar to the corresponding curve for C^{14} incorporation, except that in every case the fluctuations in P^{32} incorporation were less than the corresponding fluctuations in C^{14} incorporation.

Experiment 3.—Food was found in the stomachs of the animals sacrificed at 6, 12, 24, 30, and 48 hours after the resumption of feeding, but in the animals sacrificed at 18 hours the stomachs were empty, indicating that the animals had adopted a diurnal feeding pattern even on the first day of refeeding. This diurnal feeding pattern was based on an active feeding period in the morning (when refeeding was started) instead of feeding in the evening, as is normally the case. The metabolic fluctuations associated with this cyclic feeding pattern are reflected in the labeling data (Fig. 5). The three hour specific activities of labeled glycine and acid-soluble phosphate decreased rapidly after the starved animals resumed feeding, but were higher again at 18 hours, indicating that the dilution of labeled precursors by metabolic turnover was reduced when food was not being metabolized. This reduced metabolic turnover was evident in the reduced incorporation of the labeled precursors into the protein and RNA at 18 hours.


B
A
C

Cytological Results

Nucleus.—Fig. 7, showing two representative fields from livers obtained at the second day of refeeding in Experiment 2, illustrates the variation in nuclear surface which occurred at certain periods during refeeding in all the experiments. The nucleus marked "A" is relatively large and smooth, with finely dispersed chromatin; such nuclei are designated "smooth" in Table II. The nucleus marked "B" is distinctly smaller than "A", and its surface is wrinkled. The wrinkling of the surface is more apparent on direct microscopic examination than in the photograph; by careful focusing these nuclei can be shown to have a surface like that of a dried prune. This nucleus is typical of those designated "very wrinkled" in Table II. Nuclei which have a moderately wrinkled surface (nucleus "C" of Fig. 7) are so designated in the table, and nuclei which present only slight surface irregularities have been designated "slightly irregular" in Table II. A clear space is present around most of the wrinkled nuclei, and in certain sections there are numerous cells in which the nucleus is indented about a single large vacuole (Fig. 8).

The volumes of parenchymal nuclei in Experiments 1 and 2 are given in arbitrary units in Table II. The mean nuclear volumes varied with the changes in nuclear protein described by the curves in Figs. 1 and 3, but were not directly proportional to the protein content of the nuclear fraction. A classification of nuclei with respect to their surface appearance and the proportions of different nuclear groups at each interval during Experiments 1 and 2 are also given in Table II. The percentages of total wrinkled nuclei in the individual livers of Experiments 1 and 2 are given by the points at the bottom of Fig. 3. Very few wrinkled nuclei were present after 6 days' starvation, or at times during refeeding when nuclear volume and nuclear protein were at a maximum. In the second experiment, at the second day of refeeding, when the nuclear protein had decreased abruptly, the proportion of nuclei with wrinkled surface was at a maximum, more than 60 per cent of the nuclei showing at least moderate wrinkling of their surfaces. In the first experiment, a three-fold increase over the previous extremely low concentration of total wrinkled nuclei was observed at the fourth day, coinciding with the maximum in the nuclear protein; about the same proportion of wrinkled nuclei was present three days later. It is felt that shrinkage of the nuclei started on the fourth day, probably reached a maximum on the fifth or sixth day, and the majority of the nuclei had returned to

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Fig. 7 (Top). Photomicrographs of rat livers from the second experiment, day 2. This material was fixed in Carnoy's solution and stained by the Feulgen reaction. These livers contained a high proportion of wrinkled nuclei. Nucleus "A" is smooth and round, with finely dispersed chromatin, and is representative of nuclei classified as smooth in Table II. Nucleus "B" is smaller than "A", and is representative of the "very wrinkled nuclei" of Table II. Nucleus "C" is representative of the class "moderately wrinkled" of Table II. Magnification approximately 1200 diameters.
Fig. 8 (Bottom). Photomicrographs of rat livers from the second experiment, day 7. The livers on this day contained an appreciable number of cells in which the nucleus was indented about a large vacuole. In many cases a nucleolus lay adjacent to the nuclear membrane at its site of apposition to the vacuole. Material fixed in Carnoy's fixative, and stained with hematoxylin and eosin. Magnification 1000 diameters. We are indebted to Mr. A. S. Tracy of the Argonne National Laboratory for these photomicrographs.

TABLE II

Nuclear volume and condition of nuclear surface during recovery from starvation.

	Control	Starvation 6 days	Refeeding			
			1 day	2 days	4 days	7 days
Mean nuclear volume[3]						
Expt. 1	272[2]	135	186	199	221	[1]
	±32	± 9	±22	± 8	±18	
Expt. 2	—	221	288	235	242	266
		±18	±20	±26	±17	±22
Condition of nuclear surface (% of total)						
Expt. 1						
Smooth	61	94	82	82	68	70
	±11	± 5	± 4	± 2	± 5	± 2
Sl. irregular	19	5	13	14	20	14
	± 6	± 3	± 3	± 2	± 4	± 3
Sum, Smooth	80	99	95	96	88	84
Mod. wrinkled	14	2	4	3	11	10
	± 3	± 1	± 1	± 1	± 1	± 2
Very wrinkled	5	1	0	2	2	4
	± 2	± 0.5		± 1	± 1	± 2
Sum, wrinkled	19	3	4	5	13	14
Expt. 2						
Smooth	—	88	57	23	59	60
		± 2	±11	± 8	±11	± 8
Sl. irregular	—	9	26	15	30	17
		± 3	± 8	± 2	± 8	± 3
Sum, Smooth	—	97	83	38	89	77
Mod. wrinkled	—	2	13	20	11	14
		± 1	± 5	+ 7	± 6	± 4
Very wrinkled	—	1	5	43	1	9
		± 1	± 1	± 1	± 1	± 3
Sum, Wrinkled	—	3	18	63	12	23

[1] The nuclei after 7 days' refeeding in Experiment 1 were classified in tissue samples fixed in Baker's Calcium formal, rather than in Carnoy's fixative, which was used for all other tissue included in this table. The nuclear surface changes have been found to be the same with the 2 fixatives but the nuclear volumes would not be comparable.

[2] Each figure is the average of data from 4 individual rats, ± the average deviation.

[3] In arbitrary units.

the normal smooth appearance by the seventh day. An increase in total wrinkled nuclei comparable to that of Experiment 2 occurred in Experiment 4 also, and is shown in the bottom curve of Fig. 6. In another series, not reported here, in which only microscopic studies were made, a maximum in mean nuclear volume on the second day was followed during the next two days by a great increase (to about 60 per cent) in the proportion of wrinkled nuclei.

Thus the pattern of increase followed by reduction in nuclear protein and nuclear volume has occurred on different days in the several experiments reported here: on the first day in Experiment 2, on the second day in Experiment 3 and in the experiment not reported in detail here, and on the fourth day in the first experiment. It is felt that the difference in timing of this pattern is probably related to the metabolic state of the animals; the pattern itself has been similar in all the experiments.

Cytoplasm.—An almost complete disappearance of the perinuclear basophilic masses was noted during starvation. During the first 24 hours of refeeding these masses were more or less completely replaced, but the cytoplasm as a whole appeared much less dense than normal at this time. At later times the cytoplasm had a normal appearance.

DISCUSSION

Studies in other laboratories have shown that the cytoplasmic basophilia seen in liver tissue sections almost disappears during starvation or protein depletion and reappears rapidly when the animals are returned to a normal diet [4, 5, 6, 16, 19, 20, 30] and it has been suggested that this basophilic material in the cytoplasm represents a storage compartment for liver protein [4, 5, 6, 30]. The results of the present study indicate, however, that no single cell fraction constitutes as a whole a storage compartment for liver protein; this interpretation is in essential agreement with the conclusions reached by Kosterlitz in his very informative study of starvation and protein depletion [19], and by Miller [24] and Schultz [29] in their demonstrations of the differential loss of enzyme proteins during starvation.

For the following reasons it is felt that the basophilic clumps of liver represent material which in the intact cell consists partly of RNA-containing mitochondria and partly of associated submicroscopic structures which contain RNA: in the present study, the fraction containing the RNA-rich small mitochondria was the only cell fraction whose disproportionate loss during starvation and rapid restoration on refeeding paralleled the changes in cytoplasmic basophilia evident on microscopic examination. A decrease in mitochondria during protein depletion was also noted by Noel [25] in his classic cytological study of mitochondria. Furthermore, in cytological preparations fixed by Regaud's or Helly's methods, in which mitochondria

are well preserved, it has been possible to distinguish both basophilic and non-basophilic mitochondria [27]. The former are associated with poorly defined clouds of basophilic material which look more or less homogeneous under the light microscope, and they tend to a perinuclear arrangement, similar to that of the basophilic clumps characteristic of liver treated with fixatives containing acid or alcohol. Apparently these fixatives fail to preserve the structure of the mitochondria, with the result that the mitochondrial ribonucleoprotein is precipitated in amorphous masses. That the small mitochondria fraction represents a functional entity in the liver cell rather than a storage depot is suggested by the following facts: it contains RNA and phospholipid as well as protein [22], and, although it changes drastically in quantity, its composition changes little during starvation and refeeding. It possesses a number of enzyme activities [22, 27] and its components show an active uptake of labeled precursors.

It is noteworthy that liver protein and RNA did not start to accumulate immediately on refeeding. Although the liver weight and liver cell weight increased most rapidly during the first 24 hours of refeeding, there was very little increase in protein or RNA during this period. Apparently tissue constituents other than the two measured here, including glycogen [18], and probably water and lipids are accumulated in the liver before a significant increase in the protein or RNA occurs. A similar delay following stimulation to mitotic growth has also been noted [11, 26], during which lipid, and probably water and other constituents are accumulated before mitotic activity and the synthesis of nucleic acid and protein associated with such activity begin. Perhaps this delay represents a period of reorganization within the cell, during which various essential materials, including energy sources, are accumulated in preparation for rapid synthesis of protein and nucleic acid.

In the three experiments in which a nuclear fraction was isolated, a sharply defined accumulation and release of protein apparently occurred early during the period of replacement of liver protein and RNA. A less well-defined maximum in the RNA of this fraction also was noted, occurring simultaneously with the changes in nuclear protein. The accumulation of protein in the nuclear fraction was accompanied by an increase in the mean volume of the nuclei; the release was accompanied by a reduction in mean nuclear volume and a wrinkling in the surface of the nuclei. The release of protein from the nuclear fraction was accompanied by an increase of corresponding magnitude in the total cytoplasmic protein. Except for the protein apparently contributed by the nuclear fraction, the total cytoplasmic

protein increased in a straight line fashion throughout the recovery period. The sudden increase in the protein of the nuclear fraction was accompanied by a dramatic increase in the total labeling of this fraction. These increases were not accompanied by corresponding decreases in quantity or total labeling of protein in other cell fractions. These results, together with the observed changes in the size and shape of the nuclei, provide strong support for the view that we are dealing with a real production of protein in the nucleus rather than with an artefact due to improper fractionation of the tissue. The relative stability of the quantity and total labeling of the protein of the cytoplasmic particulate fractions, despite the wide fluctuations in the metabolic activity of the nuclear fraction, also supports this view.

These results suggest that the cytoplasm is capable of synthesizing cytoplasmic protein, at least during the brief period of time represented by the recovery period, and that a component is also contributed by the nucleus on occasion and is retained for some time in the cytoplasm. This interpretation of the data is in essential agreement with the demonstration that in fragments of *Acetabularia* and amebae from which the nucleus has been removed, protein synthesis continues, but for a limited time only [7, 8, 9, 31, 34].

The total RNA increased only during the periods when the nucleus seemed to be actively producing and releasing protein for the cytoplasm. Thus, during the periods of nuclear quiescence (days 4 to 7 in Experiment 1, and days 2 to 4 in Experiment 2), when the rate of increase in total liver protein dropped back to the rate characteristic of cytoplasmic protein (Fig. 3), the rate of increase in total liver RNA dropped almost to zero (Fig. 4). In this system, therefore, it appears that production of RNA in the cell is intermittent, and occurs in association with periods of protein production in the nucleus.

A detailed schema associating protein and RNA synthesis within the nucleus has been presented by Caspersson [13], based on his microspectrophotometric studies of various types of cells during protein synthesis. The methods used in the present study do not distinguish the various intranuclear structures, and therefore it is not possible to interpret the present results in terms that would allow detailed comparison with the conclusions reached by Caspersson. However, it would seem that in the system studied here, both the nucleus, as Caspersson has claimed, and the cytoplasm, as Brachet and his group [8] have claimed, play important and essentially independent roles in the synthesis of cytoplasmic protein.

SUMMARY

Cell fractions have been obtained from rat livers after 6 days' starvation, and at frequent intervals during the first week of refeeding. The livers were weighed, perfused, homogenized in 0.88 *M* sucrose, and separated by differential centrifugation into a nuclear, two mitochondrial, a microsomal, and a final supernatant fluid fraction. The fractions were analyzed for protein-nitrogen and nucleic acids, and the results were expressed as the quantities in the average cell and the average whole liver. In agreement with the results of other workers, there was no significant alteration in the DNA or cell content of these livers, and therefore, the two methods of expressing the results are comparable. Three-hour incorporation of inorganic-phosphate-P^{32} into the nucleic acids, and of glycine-2-C^{14} into the nucleic acids and protein of each fraction were also measured. The biochemical findings were correlated with the microscopical appearance of the parenchymal liver cells.

After 6 days of starvation, the livers lost about 50 per cent of their weight, with a corresponding reduction in the total protein and RNA contents. The losses of protein and RNA were proportional in the various cell fractions, except for a relatively smaller loss in the nuclear fraction, disproportionately large loss in the small mitochondria fraction. On refeeding, the weight of the liver increased about 50 per cent on the first day. In contrast, the early rise in the total protein and RNA was small. The earliest and most striking response occurred in the small mitochondria fraction, in which the protein and RNA content doubled in the first 24 hours. Early but less dramatic increases were also observed in the nuclear and microsomal fractions. Later, the protein and RNA of all fractions increased rapidly, approaching normal levels after about 7 days of refeeding. At different times during refeeding, in the several experiments described here, both the protein and RNA of the nuclear fraction increased abruptly to about twice the initial quantities and then decreased rapidly to the former levels. The increase in the quantity of protein in the nuclear fraction was accompanied by an increase in the specific activity and total labeling of the protein in this fraction. During refeeding, the cytoplasm as a whole accumulated protein in a straight line fashion except for a sudden increase which corresponded exactly in quantity and timing with the decrease in the protein content of the nuclear fraction mentioned above. Cytological study of the nuclei during these experiments showed that the mean nuclear volume followed the course

of changes observed in the protein content of the nuclear fraction, and that at the time when the protein of the nuclear fraction was at a maximum, the nuclei were large and round, with a smooth surface, while at the time when the protein of the nuclear fraction had decreased abruptly, the majority of the nuclei were smaller, with a wrinkled surface. An appreciable number of the wrinkled nuclei were indented around large vacuoles. The total RNA content of the liver increased only during periods of active change in the protein content of the nuclear fraction.

Considered together, these results suggest that in rat liver, during recovery from starvation, protein is synthesized by two processes: one is an intermittent production of protein by the nucleus, this process being accompanied by a net synthesis of RNA, and the other is a steady production of protein in the cytoplasm, independent of the fluctuations in the metabolic activity of the nucleus.

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UNTERSUCHUNGEN ÜBER DEN EINFLUSS DER POLIOMYELITISVIRUS-INFEKTION AUF DIE TEILUNG VON GEWEBEKULTURZELLEN

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Bei morphologischen Untersuchungen des Ablaufes der durch unterschiedliche Virusarten verursachten Infektion von Gewebekulturzellen (Affennierenepithel) machten wir die Beobachtung, dass in mit den verschiedenen Typen des Poliomyelitisvirus infizierten Gewebekulturen die Zellteilungen in den Anfangsstadien der Infektion ungestört ablaufen. Im Vergleich zu nicht infizierten Kontrollkulturen ist in den ersten Stadien der Infektion der Versuchskulturen der Prozentsatz der in Teilung befindlichen Zellen etwa gleich. Mit fortschreitender Nekrose der infizierten Gewebekultur sistiert die Zellteilung. Gelegentlich können jedoch auch in den fortgeschrittenen Stadien der Infektion, zu einem Zeitpunkt, in dem viele Zellen schon morphologische Veränderungen zeigen, noch ungestört ablaufende Zellteilungen beobachtet werden. Wurde nun das Verhalten der aus einem Teilungsprozess hervorgegangenen Tochterzellen weiter verfolgt, so konnten wir mehrmals feststellen, dass diese zu einem etwa gleichen Zeitpunkt nach der Teilung der Mutterzelle die für die Virusinfektion typischen morphologischen Veränderungen zeigten; was auffällig ist, da sich sonst die einzelnen Zellen einer Gewebekultur bei der Infektion auch mit Virussuspensionen hohen infektiösen Titers in Bezug auf den Zeitpunkt der Nekrose sehr unterschiedlich verhalten. Diese Beobachtungen warfen die Frage auf, ob die mit dem Poliomyelitisvirus infizierten Zellen noch eine ungestörte Mitose beziehungsweise einzelne Stadien der Mitose durchlaufen können oder ob die Infektion einer Zelle den Teilungsvorgang unterbindet. Obwohl die etwa gleichzeitig auftretenden morphologischen Veränderungen der Tochterzellen ein Hinweis darauf sind, dass die Infektion der Mutterzelle entweder vor oder in den ersten Stadien der Zellteilung erfolgt sein könnte, ist ein Beweis hierfür kaum zu erbringen, da die Nekrose der Tochterzellen oft erst mehrere Stunden nach vollendeter Teilung beginnt und daher nicht mit Sicherheit ausgeschlossen werden kann, dass die Infektion erst nach der Teilung erfolgte.

Versuche, denen diese Fragestellung zugrunde lag und in deren Verlauf

wir eine Reihe von Zellteilungen in infizierten Gewebekulturen verfolgten, zeigten, dass virusinfizierte Zellen verschiedene Stadien der Zellteilung morphologisch ungestört durchlaufen können, die Mitosen aber nicht vollenden, da die in Teilung befindlichen Zellen nekrotisch werden. Die zeitlichen Beziehungen zwischen der Infektion der Gewebekultur, dem Beginn der Zellteilungen und der anschliessenden Nekrose der Zellen lassen den Schluss zu, dass diese schon vor Beginn der Teilung virusinfiziert gewesen sein müssen.

Werden die Virusarten in Bezug auf das morphologische Geschehen, das sie an ihren Wirtszellen hervorrufen, in verschiedene Gruppen eingeteilt, so lassen sich einmal Virusarten finden, die, obwohl sie sich intrazellulär vermehren, keine lichtoptisch erkennbaren morphologischen Veränderungen ihrer Wirtszellen hervorrufen [7]. Eine andere Gruppe umfasst Virusarten, die, wie die tumorerzeugenden Viren, ihre Wirtszellen zu einer abnormalen Proliferation anregen [3]. Zu der Gruppe von Virusarten, die eine Destruktion ihrer Wirtszellen bewirken, gehören die in unseren Versuchen verwendeten verschiedenen Typen des Poliomyelitisvirus. Eine Stimulierung der Zellteilung konnten wir bei der Infektion von Gewebekulturzellen mit diesem Virus niemals beobachten.

Der Nachweis, dass sich die verschiedenen Typen des Poliomyelitisvirus in Gewebekulturzellen nichtnervalen Gewebes vermehren und eine Destruktion ihrer Wirtszellen verursachen, wurde durch Enders, Weller und Robbins [1] erbracht. Dieses gilt sowohl für Fibrocyten (Affenhoden, menschliche Uterusmuskulatur, menschlich-embryonales Haut-Muskel-Bindegewebe), als auch für epitheliale Zellen (menschliche Niere, Affenniere). Die cytologischen Veränderungen der Affennieren-Epithelzellen, hervorgerufen durch die Infektion mit dem Poliomyelitisvirus Typ 3 (Leon), sind von uns beschrieben worden [4].

Morphologische Untersuchungen über den Infektionsablauf an virusinfizierten Zellen sind, soweit das Geschehen an einer Zelle verfolgt werden soll, mit den uns heute zur Verfügung stehenden Hilfsmitteln nur an Gewebekulturen möglich. Die Kombination entsprechender Methoden der Gewebekultur mit dem Phasenkontrastverfahren erlaubt es, die strukturellen Veränderungen, welche die Wirtszellen durch die Virusinfektion erfahren, entsprechend den Grenzen des lichtmikroskopischen Verfahrens darzustellen.

MATERIAL UND METHODE

Wegen der besonderen Empfänglichkeit der Affennieren-Epithelzellen in der Gewebekultur für die verschiedensten Virusarten, verwendeten wir diese für unsere

Untersuchungen. Die Kulturen wurden entsprechend einer von Gey [2] beschriebenen Technik in Kammern gezüchtet, die eine fortlaufende Untersuchung mittels des Phasenkontrastverfahrens gestatten. War das Wachstum der Gewebekulturen ausreichend, wurden sie mit einer Virussuspension infiziert und der Infektionsablauf an Einzelzellen verfolgt. Während der Dauer der Beobachtung wurden die Gewebekulturen bei 37°C gehalten.

Gleichzeitig wurden von demselben Ausgangsgewebe Kontrollkulturen angesetzt, die, nicht infiziert, unter gleichen Bedingungen wie die Versuchskulturen gehalten wurden. Diese Kontrollkulturen wurden nach Abschluss des Versuches mit den virusinfizierten Gewebekulturen verglichen. Der Nachweis der Spezifität der Zellveränderungen durch die Viruseinwirkung wurde auf folgende Weise erbracht: Die Versuchs-Gewebekulturen wurden mit einer Virussuspension infiziert, die unmittelbar vor dem Versuch in Röhrchenkulturen einen cytopathogenen Effekt hervorgerufen hatte. Nach Abschluss des Versuches wurde das Nährmedium der Versuchskultur wiederum auf Röhrchenkulturen übertragen und hier bis zum Auftreten des cytopathogenen Effektes gehalten. Das virushaltige Nährmedium dieser Röhrchenkulturen wurde dann in der Neutralisationsreaktion mit dem entsprechenden Immunserum geprüft.

Zeigen einerseits die nichtinfizierten Kontrollkulturen ein normales Wachstum ohne Zeichen einer spontanen Zellnekrose, andererseits die Versuchskulturen wie auch die nachfolgend angesetzten Röhrchenkulturen eine ausgesprochene Zellnekrose und ein entsprechendes Verhalten in der Neutralisationsreaktion, so kann daraus geschlossen werden, dass die Zellveränderungen durch die Viruseinwirkung bedingt waren.

Die Lichteinwirkung auf die Versuchskulturen, die für die einzelnen photographischen Aufnahmen wie auch für die Beobachtung und Einstellung erforderlich war, wurde so gering wie möglich gehalten. Bei einer entsprechenden Lichteinwirkung auf Kontrollkulturen wurden die Zellteilungsvorgänge in diesen nicht beeinflusst.

BESPRECHUNG DER ERGEBNISSE

Wird eine Gewebekultur von Affennierenepithel mit einer Suspension des Poliomyelitisvirus Typ 1 (Brunhilde) von entsprechendem infektiösem Titer infiziert, und die Kultur bis zum Auftreten der ersten morphologischen Zellveränderungen beobachtet, so zeigt sich, dass diese nicht vor 5 Stunden nach erfolgter Infektion der Kultur auftreten. Es finden sich dann vereinzelt, unregelmässig verteilt, Zellen, die sich zunächst aus dem Epithelverband lösen, schrumpfen und sich abrunden. In der Folge nimmt die Zahl der morphologisch veränderten Zellen zu und etwa 36 bis 48 Stunden nach der Infektion der Gewebekultur sind morphologisch unveränderte Zellen kaum noch zu finden.

Bei der beobachteten und hier beschriebenen Zellteilungsstörung wurde das erste Bild (Abb. 1) 17 Stunden 30 Minuten nach der Infektion der

Gewebekultur mit dem Poliomyelitisvirus Typ 1 aufgenommen. In jedem Gesichtsfeld konnten Zellen gefunden werden, die sich in den verschiedenen Stadien der Nekrose befanden. Bei der Zelle, an der die gestörte Zellteilung mit der nachfolgenden Nekrose beobachtet werden konnte, traten 18 Stunden 15 Minuten nach der Infektion der Gewebekultur die ersten Anzeichen der Zellteilung, das Abblassen der Nukleolen, auf (Abb. 2). Die folgenden Stadien der Zellteilung verliefen unauffällig bis zur Telophase (Abb. 3, 4, 5, 6). Der zweite Teil der Telophase, die völlige Zelldurchschnürung, sowie die Ausbreitung der Tochterzellen und die Rekonstruktion der Kerne zur Arbeitsform war jedoch gestört (Abb. 7, 8). Während der Telophase zeigten die beiden noch nicht völlig abgeschnürten Teile der Zelle nur den Beginn einer Kernbildung, die auch während der weiteren Beobachtung nicht fortschritt. Während bei Normalmitosen die Ausbreitung der Tochterzellen und die Rekonstruktion ihrer Kerne schnell abläuft, blieb beides hier aus. Die begonnene Zelldurchschnürung wurde nicht vollendet und die nunmehr zwei dichte Kerne enthaltende Zelle rundete sich völlig ab (Abb. 9, 10), ein Befund, den wir niemals Teilungsvorgängen in nichtinfizierten Kulturen beobachten konnten.

Diese Zellteilungsstörung ist grundsätzlich verschieden von der, bei welcher es trotz vollzogener Kernteilung nicht zu einer Plasmateilung kommt. Diese Störung des Zellteilungsvorganges kann in älteren Gewebekulturen, bei Sauerstoffmangel oder auch bei einer Anhäufung von Stoffwechselprodukten relativ häufig beobachtet werden. Es kommt jedoch hier immer zu einer Rekonstruktion der Kerne zur Arbeitsform, die sich morphologisch nicht von solchen normal geteilten Zellen unterscheiden.

Betrachten wir die zeitlichen Beziehungen, so kann als Beginn der Mitose die Zeit 18 Stunden 15 Minuten nach der Infektion der Gewebekultur

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Für alle Aufnahmen wurde Phasenkontrastoptik verwendet. Objektiv (positiver Phasenkontrast): N.A. 0,75. Vergrösserung etwa 500 ×.

Abb. 1. Gewebekultur von Affennierenepithel (*M. mulatta*). 17 Std. 30 Min. nach der Infektion der Gewebekultur mit dem Poliomyelitisvirus Typ 1 (Brunhilde). Neben morphologisch unauffälligen Zellen solche in verschiedenen Stadien der Nekrose.
Abb. 2. 18 Std. 15 Min. nach der Infektion der Gewebekultur. Die Nukleolen in einer der in Abb. 1 dargestellten Zelle sind abgeblasst; Beginn der Mitose. Diese Zelle wurde weiter beobachtet.
Abb. 3. 18 Std. 30 Min. nach der Infektion der Gewebekultur. Prophase. Die Chromosomen werden sichtbar, Nukleolen nicht mehr zu erkennen.
Abb. 4. 19 Std. 15 Min. nach der Infektion der Gewebekultur. Metaphase. Bildung der Äquatorialplatte.
Abb. 5. 19 Std. 45 Min. nach der Infektion der Gewebekultur. Anaphase.
Abb. 6. 20 Std. 10 Min. nach der Infektion der Gewebekultur. Telophase. Beginnende Einschnürung des Cytoplasmas.

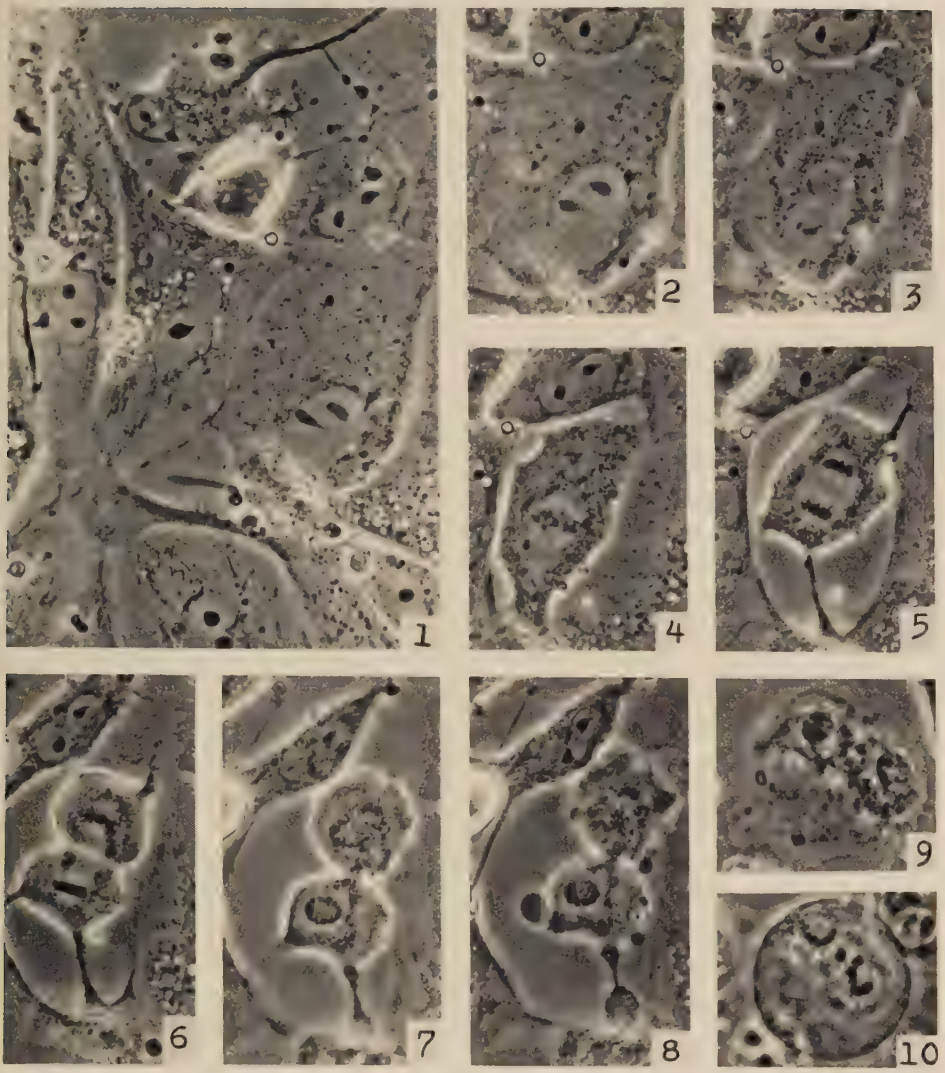

Abb. 7. 20 Std. 40 Min. nach der Infektion der Gewebekultur. Die Einschnürung des Cytoplasmas ist weiter fortgeschritten. Beginnende Kernrekonstruktion in den fast abgeschnürten Teilen der Zelle.

Abb. 8. 20 Std. 50 Min. nach der Infektion der Gewebekultur. Cytoplasmatische Bläschen an der Zelloberfläche, die für dieses Stadium der Zellteilung charakteristisch sind. Die Durchschnürung des Cytoplasmas ist gegenüber Abb. 7 nicht weiter fortgeschritten.

Abb. 9. 22 Std. 15 Min. nach der Infektion der Gewebekultur. Die Durchschnürung des Cytoplasmas ist nicht vollendet worden, die nunmehr zwei dichte, abnormale Kerne enthaltende Zelle ist völlig abgerundet. Einstellung auf die beiden Zellkerne.

Abb. 10. 23 Std. 45 Min. nach der Infektion der Gewebekultur. Einstellung auf die Zellperipherie, die, wie schon in Abb. 9, völlig abgerundet ist.

angenommen werden (Abb. 2). Die ersten morphologisch erkennbaren Störungen der Zellteilung traten 20 Stunden 50 Minuten nach der Infektion der Gewebekultur auf (Abb. 8). Das Zeitintervall zwischen diesen beiden Beobachtungen beträgt 2 Stunden 35 Minuten. Dieser Zeitraum ist aber zu kurz, um morphologische Veränderungen an einer Affennieren-Epithelzelle durch eine Infektion mit dem Poliomyelitisvirus Typ 1 auszulösen, selbst wenn die Gewebekultur mit einer Virussuspension von hohem infektiösen Titer infiziert worden wäre. Es erscheint uns somit gerechtfertigt anzunehmen, dass die Zelle schon vor Beginn ihrer Teilung mit dem Virus infiziert gewesen sein muss. Wird weiter vorausgesetzt, dass die Anaphase zeitlich etwa in der Mitte der Gesamtablaufszeit bei Normalmitosen liegt, so hätte die Rekonstruktion der Tochterzellen zur Arbeitsform etwa 21 Stunden 15 Minuten nach der Infektion der Gewebekultur beendet sein müssen, zu diesem Zeitpunkt war die Zelle aber bereits abgerundet und nekrotisch.

Die Annahme, dass, neben Infektionen mit Virusarten aus der Gruppe der tumorerzeugenden Viren, virusinfizierte Zellen grundsätzlich einen Teilungsvorgang durchlaufen können, wird durch Beobachtungen gestützt, dass bei Virusarten, die Einschlusskörperchen in ihren Wirtszellen bilden, gelegentlich in derselben Zelle gleichzeitig Einschlusskörperchen und Zellteilungsformen gefunden wurden [5]. Es handelt sich hierbei aber um fixiertes und gefärbtes Material, sodass Aussagen über den Ablauf der Zellteilung nicht möglich sind.

ZUSAMMENFASSUNG

Zellteilungen in poliomyelitisvirus-infizierten Gewebekulturen (Affennierenepithel) wurden untersucht. Es konnte gezeigt werden, dass virusinfizierte Zellen noch einzelne Stadien der Teilung durchlaufen können, ehe sie nekrotisch werden.

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A SIMPLE TECHNIQUE FOR THE ELECTRON MICROSCOPY OF FRESH ASCITES TUMOUR CELLS

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For examination in the electron microscope, tissue cells must be thin enough to allow penetration by the electron beam. Porter, Claude and Fullam [41] were the first to recognise that cells in tissue culture would be suitable for electron microscopy on account of the tendency they exhibit to flatten out to extreme thinness when in contact with a supporting glass surface. These workers developed a technique in which cells were grown in roller tubes on a cover slip coated with a plastic membrane; after fixation the membrane with cells adhering was transferred to a specimen supporting grid of the electron microscope. They and their collaborators have used the method to study many different types of cell as well as the effects of various fixatives on cells [15, 44, 45, 42 and 43]. The same basic procedure has been adopted by other investigators [3, 31, 35, 46, and 8] but usually with less elegant results.

In recent years great advances have been made in the application of suitably modified conventional histological methods to the cutting of sections of cells sufficiently thin for electron microscopy and the results have contributed enormously to current knowledge of cell ultrastructure [36, 37, 47, 48, 51, 49 and 50]. However, as Porter has pointed out [40] study of ultrastructure in the whole cell can be uniquely valuable and this claim he has recently well illustrated [38].

It was thought therefore, that a method for preparing whole cells for electron microscopy which avoided the labour and uncertainty of the tissue culture technique would be worth developing.

It has been shown by Bessis [9, 10] and Bessis and Bricka [11, 12] that freshly obtained leucocytes could be rendered suitable for electron microscopy by making use of their great motility on flat surfaces; for, these cells will move about, spreading thinly in the process, if incubated on a slide coated with a plastic membrane. The method has been used by others for the study of white blood cells, both normal [5, 7, 30] and leukaemic [6, 33,

34]. It was considered that a similar technique might prove applicable to the single dispersed cells occurring in the ascites forms of transmissible animal tumours since it has long been known that malignant cells are capable of amoeboid movements [13, 27] and recent studies have shown that their motility is considerably greater than that of comparable normal cells [18, 20]. Some additional evidence that ascites tumour cells might be induced to flatten out like leucocytes sufficiently well for good electron microscopy was afforded by the reports of attempts made to work with the cells in ascitic fluids complicating a malignant reticulosis of mice and advanced cases of human malignant disease [34, 25].

The present communication describes the technique which has been devised for preparing cells from the ascites forms of mouse Sarcoma 37, mouse Krebs II adenocarcinoma and the Rous No. 1 fowl sarcoma for examination in the electron micrscope.

MATERIALS AND METHODS

Tumours.—(1) Ascites Sarcoma 37—mouse Sarcoma 37 was maintained as an ascites tumour as defined by Klein [26]. It was passed in series by intraperitoneal inoculation in mice of whole ascitic fluid in 0.1 ml volumes.

(2) Ascites Krebs II carcinoma—mouse Krebs II adenocarcinoma was maintained in the same way as Sarcoma 37.

(3) The ascites form of the Rous sarcoma—the Rous No. 1 fowl sarcoma used was derived from that first introduced into the Bland-Sutton Institute in 1925. For the present work minced Rous sarcoma tissue was inoculated intraperitoneally in chickens to give ascitic fluid containing free virus and large numbers of single tumour cells in the way first reported briefly by Epstein [22]. The condition consisted of a sarcomatosis peritonei caused shortly after inoculation when the tumour cells settled and multiplied on the visceral peritoneum and an ascites which developed some days later as a result [23]; it was maintained by serial passage of the fluid.

For each passage a bird with abdominal swelling (about 10 days after inoculation) was killed by cervical dislocation; the tumour ascites was drawn off and was inoculated intraperitoneally in 4 ml volumes into a group of chickens. For these inoculations the abdominal wall was pierced by a wide bore needle half way between the lower end of the sternum and the spine of the right pubis and a blunt ended canula with subterminal holes was passed through this needle into the abdominal cavity; the ascitic fluid was injected down the canula which was then withdrawn from the needle thus avoiding contact with the abdominal wall and eliminating tumour growth in the needle track.

Animals.—(1) The mouse tumours were passed in young adult albino mice obtained throughout from the same stock.

(2) The ascites form of the Rous sarcoma was passed in pedigreed Brown Leghorn fowl from the Poultry Research Centre, Edinburgh.

Preparation of materials.—De-ionised water was prepared by passing distilled water through a column containing acidic and basic ion exchange resins (Amberlite "Monobed" Resin MB-1 of British Drug Houses, Poole, England). New hard or flint glassware was kept specially for the work, it was washed with soap and tap water, soaked for 2 days in de-ionised water at 4°C and dried thoroughly before storing ready for use. Sterile balanced saline solution was prepared as described by Earle [19] except that de-ionised water was used and sterilization was carried out by filtration through a Chamberland L3 candle. The saline solution was stored in 5 ml volumes in sealed ampoules at 4°C.

A hot air oven or de-ionised water at 100°C were employed to sterilize apparatus used in preparing the saline solution; autoclaving was avoided at all stages since it has been found that traces of metallic ions become deposited on glassware sterilized in this way, probably being carried over in the steam [17].

The cell preparations were made on 3″ × 1 ½″ glass slides coated before use with a thin plastic membrane. The slides were dipped in a 0.4 per cent solution of Formvar (obtained from Shawinigan Resins, Ltd., London) in freshly distilled ethylene dichloride and then left to drain for at least 18 hours at an angle of 55° to the horizontal, open to the air at room temperature to allow complete evaporation of the ethylene dichloride, but protected from dust. The slides were scored with a cross on the underside ¾″ from the bottom to mark the area on which the cell preparation was to be made, thus ensuring that membranes of approximately the same thickness were used from each slide. Under certain atmospheric conditions the cooling of the slides during evaporation of the ethylene dichloride caused moisture to condense in the Formvar films making them milky and unsuitable for use. To avoid this the slides were warmed to about 56°C before coating.

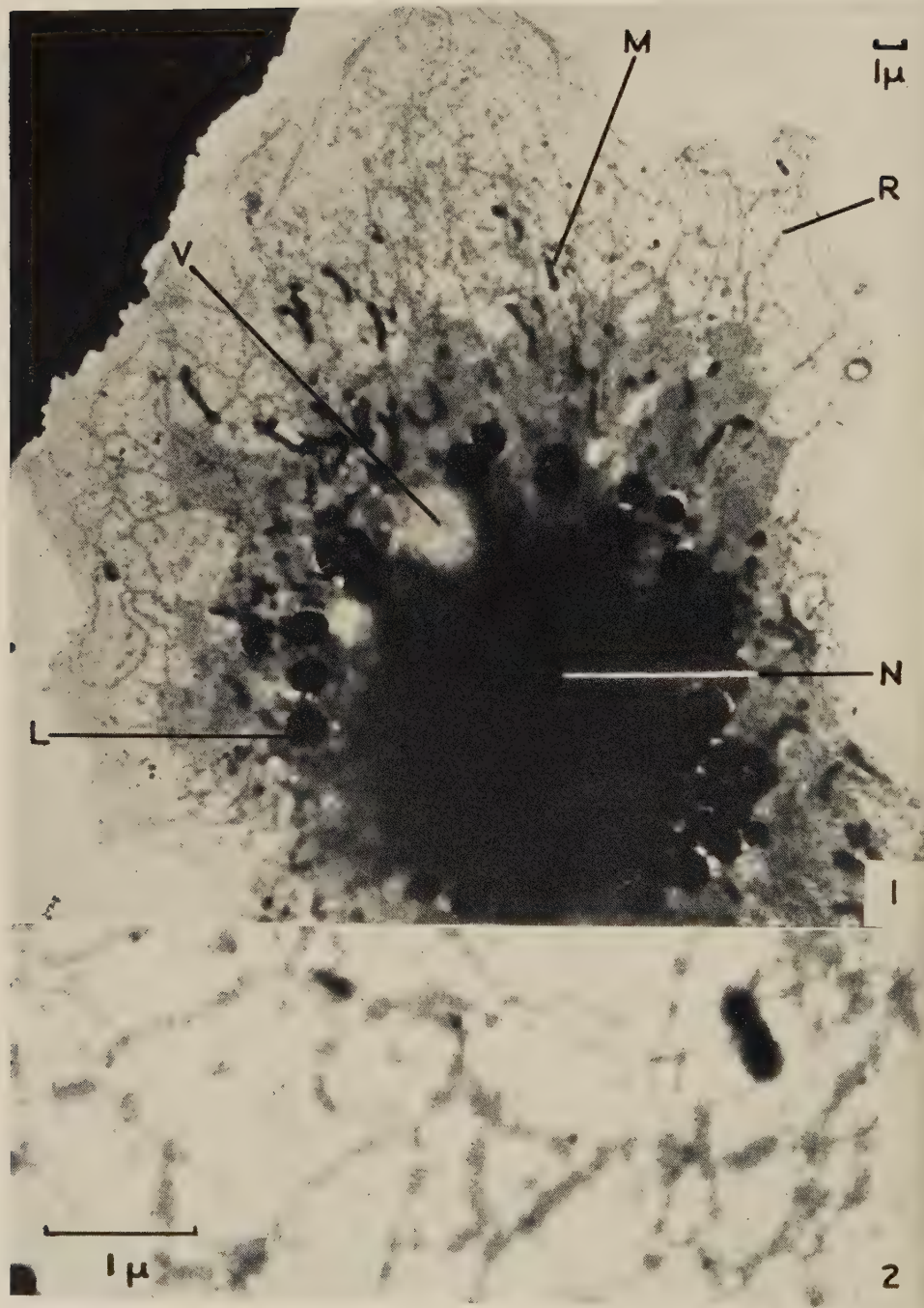
Preparation of tumour cells.—Everything which was to come into contact with the cells was warmed to 37°C in an incubator. It was found that if the Formvar coated slides were warmed in a container having an atmosphere of high humidity subsequent stripping of the membrane from the slides was facilitated.

Sarcoma 37 ascites cells.—Three to 4 days after inoculation a mouse was killed by cervical dislocation and its ascitic fluid, having usually about 50,000 tumour cells per cu.mm, was drawn off. Enough was then run into a 3″ × ½″ tube of diluent to give a 1 in 50 dilution. The diluent consisted of 1 part of serum (pooled from 3 to 5 mice) in 3 parts of Earle's balanced saline solution.

Krebs II ascites cells.—These were suspended in diluent in the same way as were the Sarcoma 37 cells. It was found best, however, to make use of an ascites which had developed for 4 to 5 days after inoculation, at which time the tumour cells numbered about 100,000 per cu.mm.

Rous ascites cells.—A bird with recognisable abdominal swelling following intra-abdominal inoculation of ascitic fluid 8 to 10 days previously was selected and 7 ml of blood were withdrawn by cardiac puncture. The blood was defibrinated by the method of Eppes and Ludovic [21] and the serum was used to make the diluent described above. The bird was then killed by cervical dislocation and its ascitic fluid was drawn off and treated as was Sarcoma 37 ascites. The number of tumour cells was usually in the region of 20,000 per cu.mm.

Experimental procedure.—The diluent containing ascites cells was run into a series of hollow ground slides so as to fill their cavities. The slides measured 3″ × 1 ¼″


M
R
V
N
L
1
1 μ
2
1 μ

and each had a centrally placed cavity ¾″ in diameter; before use a thin film of vaseline was smeared in a ring round the edges of the cavities. When each cavity was filled it was closed by pressing a Formvar coated slide over it into firm contact with the vaseline ring in such a way that its coated surface was in contact with the diluent and cells and its cross lay over the centre of the cavity.

The slide assemblies were then inverted to bring the Formvar slides underneath and were incubated at 37°C for about 20 minutes. On inversion the ascites tumour cells in the diluent sank down and came to rest on the Formvar on which they spread out and moved about. The progress of this phenomenon was followed with a phase contrast microscope fitted with a warm stage and when sufficient cells were judged to be well spread the slide assemblies were again turned bringing the Formvar coated slides uppermost. They were incubated in this position for a further 2 minutes to allow unspread cells and any débris present to fall away from the Formvar. Then the slide assemblies were opened and each Formvar coated slide with spread cells on it still bathed in an adhering film of diluent was pressed Formvar side down on to vaseline smeared round the rim of a Maximow chamber [32] made of perspex and containing 1.5 ml of 2 per cent osmium tetroxide solution in distilled water. Fixation of the cells in the osmium vapour was continued for about 21 hours; a relatively large volume of fixing solution was used in order to give a moisture saturated atmosphere below the film of diluent covering the cells, thus ensuring that the diluent did not evaporate during fixation.

At the end of fixation the slides were removed from the fixing chambers and were placed horizontally with their Formvar coated sides down in a rack and were washed in distilled water for 24 to 48 hours, the water being kept gently running for as much of this time as possible.

When the period of washing was completed a number of small areas of Formvar membrane were selected at random from the region of each slide round the scored cross, this region being covered with large numbers of spread fixed cells. Each area of Formvar membrane selected was stripped from the slide with its cells adhering to it and mounted on a specimen supporting grid of the electron microscope by a technique described elsewhere (24). The specimens were then dried *in vacuo* over P_2O_5 preparatory to examination.

General considerations.—The ascites tumours were passed with strict aseptic technique; tests for the presence of contaminating bacteria were negative.

Cell counts were performed on the ascitic fluids after they had been diluted 1 in 20 in 1 per cent aqueous eosin in a white cell pipette and placed in an improved Neubauer counting chamber (of Hawksley Ltd., London). The tumour cells were

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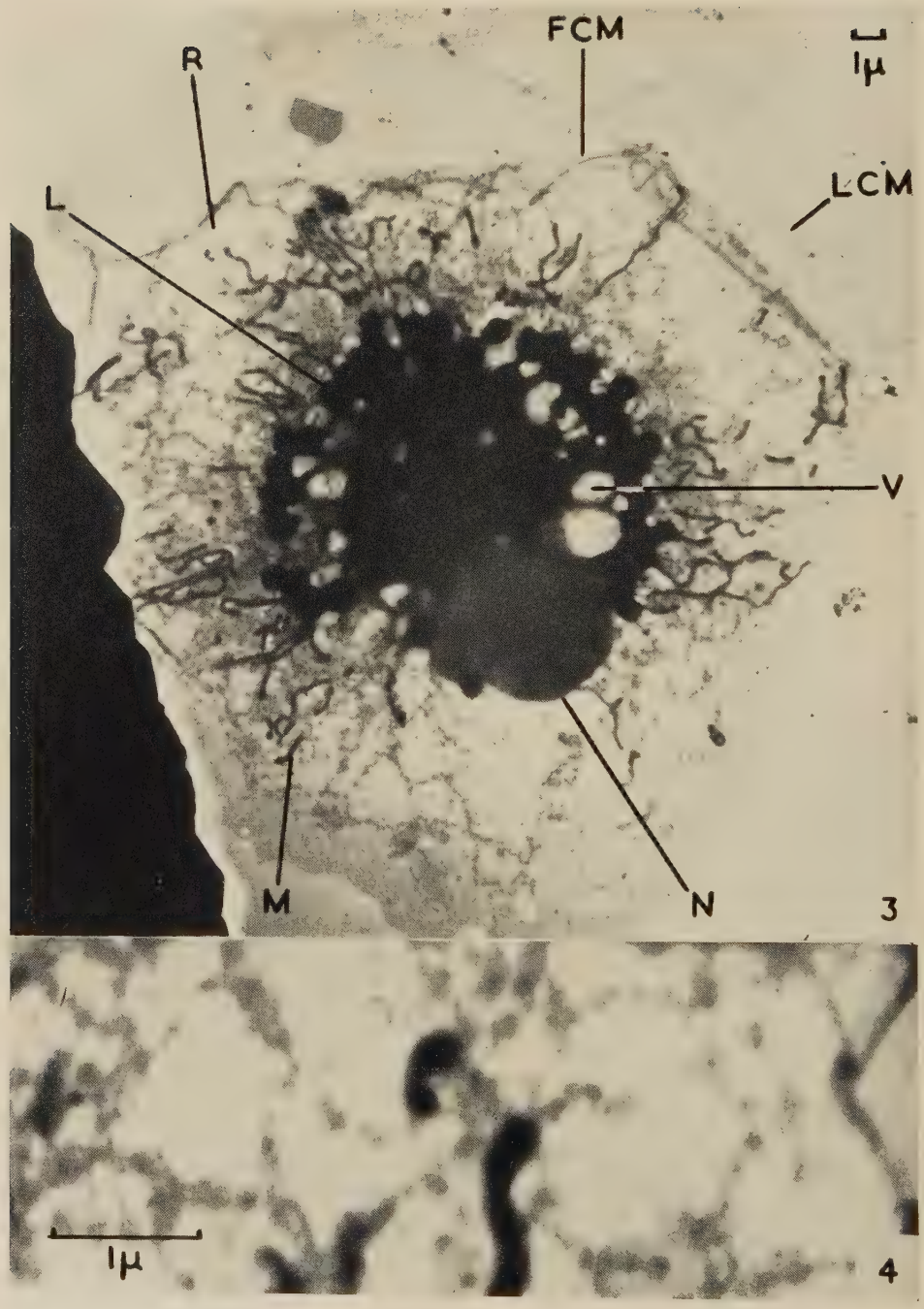
Fig. 1. Electron micrograph of a Sarcoma 37 ascites tumour cell; about two thirds of the cell are included. The cell was allowed to flatten on a Formvar coated slide for 20 minutes after removal from a tumour bearing mouse and was then fixed in osmium vapour for 20 hours. The very large electron opaque nucleus (*N*) is surrounded by large osmiophilic lipoid bodies (*L*); a zone of short rod-shaped mitochondria (*M*) and two vacuoles (*V*) can also be seen. An endoplasmic reticulum (*R*) radiates through the cytoplasm. The edge of a grid bar lies across the top left hand corner of the field. Accelerating voltage 60 kV. Magnification × 4000.

Fig. 2. Details of the endoplasmic reticulum from the cell shown in Fig. 1. A short rod-shaped mitochondrion is included in the field. Accelerating voltage 60 kV. Magnification × 20,000.


FCM
1μ
R
L
LCM
V
M
N
3
1μ
4

differentiated from other cells on the basis of their greater size since this has been shown to correspond well with accurate figures arrived at by differential counts made on stained preparations [4, 39].

The dilution factor used for the ascites fluids was that found empirically to give the most convenient density of spread cells on the Formvar with hollow ground slides of the capacity employed. With these slides the area of Formvar which could be covered with spread cells was sufficient to give 5 or 6 samples of membrane for mounting on 3 mm diameter specimen grids.

The examination of the material when prepared was carried out using a Philips EM-100 electron microscope of the new type 11980/23 embodying the new high resolution objective lens; the objective aperture of 30 μ diameter was found to give the best image contrast. All the electron micrographs were taken using an accelerating voltage of 60 kV.

RESULTS

Phase contrast microscopy.—A change in shape of the tumour cells from a round to a spindle form was always observed when they were brought into contact with a firm surface on which to grip. The cells spread out and became suitable for electron microscopy early in this change of shape and not simply when they began to move about, for, amoeboid movement was seen in the cells directly the transformation had started and it continued after the spindle shape had developed. When the cells were left until the change was complete the resulting spindle shaped cells were found to be too thick for penetration by the electron beam.

Spreading of the cells usually commenced 5 to 10 minutes after the slide assemblies were set up. The number of cells changing shape and spreading out varied with the three tumours used. Almost all Rous ascites cells flattened well and rapidly whereas only about 50 per cent of Sarcoma 37 cells behaved in this way; with the Krebs II carcinoma cells an even smaller percentage still underwent a change of shape. The fowl cells tended to spread more thinly than those of the mouse tumours.

Fig. 3. Electron micrograph of a Rous ascites tumour cell which had been allowed to flatten for 26 minutes before fixing in osmium vapour for 22 hours. The nucleus (*N*) is small as are the profuse lipoid granules (*L*) which fill the centre of the cell and surround a number of vacuoles (*V*). The mitochondria (*M*) are long and filamentous and an endoplasmic reticulum (*R*) lies between them in the cytoplasm. The cell membrane has become torn at the extreme edge of the cytoplasm at the top of the field and is well seen since the upper portion is folded back (*FCM*) whilst the lower has remained spread on the Formvar (*LCM*). The edge of a grid bar lies on the left of the field. Accelerating voltage 60 kV. Magnification × 4000.

Fig. 4. Detail of the endoplasmic reticulum from the Rous cell shown in Fig. 3. Part of a filamentous mitochondrion is included in the field. Accelerating voltage 60 kV. Magnification × 20,000.

Electron microsopy.—About half the samples of membrane taken at random from any one series of slide assemblies were found to have well prepared cells on them in large numbers; on the rest the proportion of such cells tended to be lower owing to patchiness in the distribution of good fixation.

The electron micrograph reproduced in Fig. 1 shows a tumour cell from a Sarcoma 37 ascites whilst that in Fig. 3 shows a Rous sarcoma ascites cell. The mouse tumour cell is considerably larger than that of the fowl tumour and this was the case not only with most Sarcoma 37 cells but also with cells of the Krebs II carcinoma.

The same structures were found in each type of cell examined, namely, the nucleus, osmiophilic lipoid granules, mitochondria, vacuoles and an endoplasmic reticulum. As the two examples indicate (Figs. 1 and 3) these structures had a characteristic form in the cells of each type of tumour. In the mouse cells the nuclei were large and dense, the lipoid granules were large and abundant in the Sarcoma 37 cells but rare in the Krebs II cells and the mitochondria of both were short and rod-shaped; large vacuoles were frequently seen in the Sarcoma 37 cells. The fowl tumour cells on the other hand had small nuclei, a profusion of small lipoid granules, plentiful filamentous mitochondria and fairly numerous central vacuoles. In each type of cell the size and number of the lipoid bodies increased when adverse changes were allowed to take place in the diluent in the slide assemblies, for example, oxygen lack or cooling, The same increases were found to occur where the *in vivo* environment of the cells deteriorated, i.e. where cells were taken for examination from moribund animals.

In Fig. 3 the cell membrane is well demonstrated since it has become torn at the extreme edge of the cell and the upper layer is folded back leaving the lower part still spread out on the Formvar. Similar appearances have been encountered in all three types of cell examined.

Figs. 2 and 4 show details at higher magnification of the endoplasmic reticulum from the mouse cell and fowl cell respectively.

DISCUSSION

The technique described in this paper allows large numbers of preparations of cells to be made with relative ease and its simplicity has permitted the trial of many different modifications at each stage. The steps described here are those which have been found to be important if the best results are to be obtained; the following points are thought to require comment.

Great efforts have been made throughout the work to reduce the likelihood

of toxic ions coming into contact with the cells. Even traces of such ions interfere with the ability of the cells to spread widely and since good spreading is the key to satisfactory electron microscope preparations with this technique, the precautions described are of importance.

Serum was chosen as a constituent of the diluent rather than cell-free ascitic fluid since different samples of the latter varied widely especially in their tendency to clot. Using serum, the amount of protein in the diluent could be standardised and reduced to a low level provided that the Earle's saline solution and glassware were prepared as has been described. With this diluent, washing of the cells before fixation was not required. Whenever washing of the spread unfixed cells was attempted shrinkage and distortion resulted. Martin and Tomlin [31] working with tissue culture cells have reported similar difficulties although in the technique of Porter and his co-workers [40] such cells were always washed before fixation. It is suggested that the firmness of the adhesion of the cells to a Formvar membrane may influence the occurrence of this shrinkage and retraction; the spread tumour cells in the experiments reported here were allowed only some 20 minutes in which to gain a grip on the Formvar whilst tissue culture preparation have usually been left in roller tubes for 1 to 2 days. The method of fixing the tumour cells in osmium vapour whilst upside down in a hanging film of low protein diluent, together with the prolonged washing upside down after fixation, ensured that the preparations were free from débris and coagulated protein.

With regard to the spreading of the cells, the change from the free round form to a spindle form has been studied by Craigie [16] and Lasnitzki [28, 29] in the ascites cells of mouse tumours and the present work confirms that such cells possess a bimorphism conditioned by their environment.

The fact that the same dilution factor was suitable for each of the three types of ascites cell despite the differences in their average total cell counts, reflects the difference in the proportion of cells which changed shape and spread out. It may be that this difference in the behaviour of the three tumours can be accounted for by different percentages of "paramorphic" cells [16] in their cell populations.

The finding during the present work that fowl cells spread better for electron microscopy than mouse cells confirms a similar difference observed by Claude [14] working with avian and mammalian cells grown in tissue culture. It is considered that not only the smaller size of the fowl cells but also a greater inherent ability to flatten out and become thin makes them easier to examine by electron microscopy.

Although the ascites form of the Rous sarcoma does not conform to the usually accepted definition of an ascites tumour [26] but consists of an ascites containing tumour cells accompanying a sarcomatosis peritonei, it was only required here as a source of single tumour cells in fluid and not specifically as a true ascites tumour. It is of interest to note that the Rous ascites sarcoma has maintained its characteristic pathology through 43 fluid passages and that another ascites strain of this tumour recently established elsewhere [1] has been found to behave in a similar way [2].

In considering the structures seen with the electron microscope in the three types of tumour cell and described above, a number of points emerge. The structures are the same as those which have been reported in all types of cell apart from blood cells so far examined by electron microscopy. It is considered important in this connection that the ascites tumour cells were always fixed within half an hour of removing them from a tumour bearing animal whereas cells previously examined by tissue culture techniques have usually been *in vitro* for more than 24 hours. The endoplasmic reticulum for example is therefore clearly not an abnormal manifestation resulting from a prolonged stay in an unnatural environment.

With regard to the fact that no electron opaque particles have been seen in the Rous ascites cells similar to those reported by Claude, Porter and Pickels [15] in cultured Rous cells, it must be remembered that the tumour strain used in the present work has been maintained at the Bland-Sutton Institute for more than 30 years as a separate offshoot of the original Rockefeller Rous No. 1 fowl sarcoma in which the particles were reported. The demonstration of the cell membrane through its becoming folded back as in the Rous cell shown in Fig. 3 is thought to arise from tearing at the cell edge at some stage after fixation as a result of the particular methods of handling the cells which have been employed.

In conclusion, the finding that in the types of ascites tumour cell examined all the features are present hitherto reported in cultured cells subjected to electron microscopy, is held to justify the development of the technique described. The present work was undertaken not as an electron microscope study of tumour cells *per se* but in order to evolve a simple reliable method for making cell preparations in quantity. The technique reported provides readily prepared cells suitable as test material for such work as the investigation of the endoplasmic reticulum under varying conditions, the study of tissue fixatives, or the development of selective electron opaque stains.

SUMMARY

A technique for preparing freshly collected cells from ascites tumours for electron microscopy is described. The method takes advantage of the fact that such cells spread and flatten out to extreme thinness a few minutes after coming into contact with flat surfaces under suitable conditions. The more important steps in the technique are discussed. The method is considered useful in that it is a simple and reliable means of preparing whole cells as test material in electron microscopy.

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CYTOCHROME OXIDASE ACTIVITY IN MITOCHONDRIA DURING AMPHIBIAN DEVELOPMENT

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In adult mammalian organisms, cytochrome oxidase and succinoxidase, as well as a number of other enzymes, are localized in the mitochondria of the cell (see [18, 17, 4, 14] for reviews of the literature). There is some evidence that also in embryonic cells and tissues these enzymes are associated with mitochondria. The relevant data, however, are meager and to some extent contradictory. Shen [19] found that cytochrome oxidase and succinoxidase are localized in the mitochondria of developing rat muscle, at least from the fifteenth day of gestation on. In addition, Recknagel [16] has reported the localization of cytochrome oxidase in mitochondria in the ovarian eggs of *Rana pipiens*. In the egg of the sea urchin, *Arbacia punctulata*, on the other hand, it has been claimed that cytochrome oxidase and succinoxidase are found in particulate matter which is non-mitochondrial in nature [10, 11, 5]. Indeed, Hutchens *et al.* [10] showed that only 1 per cent of the cytochrome oxidase activity of the unfertilized *Arbacia* egg could be found in the mitochondria. It has been suggested that cytochrome oxidase has an extra-mitochondrial locus in the sea urchin embryo during the early part of development [12, 6, 7] and that later, beginning with the mesenchyme blastula stage, when the mitochondria begin to increase in number, the enzyme becomes incorporated into them [6, 7]. Data obtained recently from isolated fractions of *Paracentrotus lividus* eggs and embryos do not support the view that cytochrome oxidase has an extra-mitochondrial location. Active enzyme can be demonstrated neither in the microsomes nor in the soluble fraction; it is present, however, in large, centrifugable granules that have the characteristics of mitochondria [22]. It seems reasonable to assume, therefore, that the increase in enzyme activity that occurs during sea urchin development represents synthesis within the mitochondria.

In view of the lack of agreement in the work cited above, and also because no systematic study has so far been made on the relationship of cytochrome oxidase and succinoxidase to mitochondria throughout a major part of the

period of embryonic development, the present investigation was undertaken. We have sought the answers to two questions: (1) Are these enzymes associated with mitochondria at all stages of development, or do they at certain times have an extra-mitochondrial location in the cell? (2) Is the increase in enzyme activity which has been demonstrated to occur in the embryo during development [1, 2] brought about solely by multiplication of mitochondria, or are maturative or differentiative processes in the mitochondria responsible, at least in part, for the changes observed?

MATERIAL AND METHODS

Eggs and embryos of *Xenopus laevis*, permitted to develop normally at 18°C, were used. Twenty embryos, carefully selected so as to be of uniform stage, were freed of jelly and vitelline membranes. They were then homogenized in a medium consisting of 0.28*M* sucrose containing 0.0001*M* versene [20] and 0.012*M* phosphate buffer at pH 7.4. Homogenization was done in an ice bath, and all subsequent handling of the homogenate, except the enzyme assays, was done in a low-temperature room.

The homogenate was diluted to 400 μl. One hundred microliters were used for enzyme assays and Kjeldahl nitrogen determination [3]; the remaining 300 μl were fractionated to yield the following: yolk, pigment, mitochondria, microsomes, supernatant, and washings. The particle fractions from homogenates of ovarian eggs were isolated by differential centrifugation in a refrigerated International Centrifuge (head no. 295). The homogenates of developing stages were fractionated by means of a microcentrifuge having an angle head and 12 tubes of 0.5 ml capacity.[1] By working in a cold room, it was possible to keep the heating effects, due to high speed centrifugation, within reasonable limits. The technical details relating to the preparation of the fractions are summarized in Table I.

In some experiments, the enzyme activities of all fractions, save the washings, were determined. In most cases, however, only the homogenate and mitochondria were used, since our main interest lay in a comparison of their specific activities and determination of the relative amount of the enzyme in the mitochondrial fraction. In a few experiments, mitochondria and microsomes were isolated by centrifuging egg homogenates in a sucrose-diodon density gradient after the technique described by Holter, Ottesen, and Weber [9].

Cytochrome oxidase activity was determined at 20°C by means of the Cartesian diver technique. In some cases, succinoxidase activity was also measured. Four-tenths microliter of alkali was first placed in the diver bulb; next 0.4 μl cytochrome *c*-substrate mixture was introduced as a side drop on the previously siliconed diver neck, and finally 0.6 μl of enzyme suspension was pipetted into the neck as a seal. Oil seal and neck seal were then introduced as usual. After a period for temperature equilibration, usually 10 minutes in length, the enzyme and substrate were mixed, and the reaction was followed for approximately an hour during which the oxygen

[1] This microcentrifuge was designed by Mr. Ole Dich, 18 Holmevej, *Brondby Strand*, Denmark. It can now also be supplied with a head that accommodates horizontally rotating tubes.

TABLE I

Centrifugal forces used to isolate particle fractions from homogenates.

Fraction	Centrifuge time	Centrifugal force
Ovarian eggs		
Yolk	2 × 10 min.	250 × G
Pigment	3 × 5 min.	1,000 × G
Mitochondria	20 min + 30 min.	7,500 × G
Microsomes	90 min.	20,000 × G
Developmental stages and tadpole liver		
Yolk, pigment and debris	2 min.	120 × G
Mitochondria	15 min.	17,500 × G

uptake was at a linear rate. Readings were taken at 5- or 10-minute intervals. Corrections for endogenous oxygen uptake of enzyme preparations and autoxidation of substrate were always made. The total nitrogen of the enzyme preparations was determined [3] on appropriately diluted aliquots, and the specific activity (Q) of the enzyme was thus expressed on a nitrogen base.

An elaborate series of preliminary experiments was run in order to determine (a) the optimum concentration of substrate, (b) the optimum concentration of homogenate or cell fraction so as to insure a linear relationship between enzyme concentration and activity, and (c) the most suitable diluent for the enzyme preparations.

Para-phenylene diamine (final concentration 0.04M) and sodium succinate (final concentration 0.05M) were used as substrates; cytochrome c was added to yield a final concentration of $5 \times 10^{-4}M$, and the concentration of the enzyme was adjusted so that the pressure change in the divers was approximately 120 mm per hour. Homogenate or homogenate fractions were diluted with a solution consisting of equal parts of amphibian Ringer's solution and phosphate buffer at pH 7.4. We found that with this dilution medium, but not with sucrose-versene solution or distilled water, the rate of oxygen uptake was constant for 60 minutes or more.

RESULTS AND DISCUSSION

In this paper, only the data for cytochrome oxidase will be reported in detail. The results of succinoxidase determinations are qualitatively similar to those of cytochrome oxidase. They differ only quantitatively in that succinoxidase activity is about one-tenth that of cytochrome oxidase.

In a few preliminary experiments, the cytochrome oxidase activity of mitochondria which had been isolated by centrifugation in a sucrose-diodon

Table II

Comparison of the specific activity of mitochondria obtained from ovarian eggs by two different methods.

Method of preparation	μg N/diver	Q
Differential centrifugation	0.40	668
,, ,, ...	0.20	660
Density gradient	0.17	518
,, ,, ...	0.17	530
Unfractionated homogenate . . .	2.97	68

density gradient [9, 23] was compared with that of mitochondria, from the same homogenate, obtained by differential centrifugation. One such test is shown in Table II. The cytochrome oxidase activity of gradient-isolated mitochondria proved to be somewhat lower than that of mitochondria prepared in the usual fashion by differential centrifugation. This may be due, among other things, to (1) a slightly deleterious effect of the gradient medium on the enzyme, or (2) the inclusion of a contaminant (perhaps diodon) in the mitochondrial fraction that contributes to the total nitrogen but possesses no cytochrome oxidase activity. Because of these results, we confined our observations to cell fractions that were isolated by differential centrifugation.

Localization of an enzyme in mitchondria can be regarded as reasonably well established if the specific activity of the mitochondria is much greater than that of the tissue homogenate and if the bulk of the activity can be recovered in the mitochondrial fraction (4). In order to test the adequacy of our technique, involving as it did fractionation of relatively small amounts of starting material, we performed an experiment using livers of old *Xenopus* larvae at the third-form tadpole stage of development. The results of this experiment are summarized in Table III and show that in fully differentiated *Xenopus* liver, as in adult mammalian liver, cytochrome oxidase is localized in mitochondria.

Table IV summarizes an experiment on the distribution of cytochrome oxidase in various fractions obtained from ovarian egg homogenates. The data show that most of the enzyme is localized in the mitochondrial fraction, but the proportion of the total homogenate activity found in the mitochondria is relatively less than with liver. In general, with older embryos, the amount of succinoxidase and cytochrome oxidase in the mitochondrial fraction was found to be somewhat greater than in ovarian eggs. The average figure was approximately 70 per cent for cytochrome oxidase and 75 per cent for

TABLE III

Distribution of protein nitrogen and cytochrome oxidase in tadpole liver.

Fraction	Total *N*		Cyt. ox.		
	μg	per cent	*Q*	Total	per cent
Homogenate	55.1	(100)	167	9250	(100)
Tissue debris	[1]		0	0	0
Mitochondria	20.3	37	437	8850	96
Supernatant	21.6	39	0	0	0
Washings	10.8	20	—	—	—
Total	52.7	96			

[1] Too small to measure. The homogenate was freed of most of the tissue debris (unbroken cells and connective tissue) before fractionation was begun. This was accomplished by allowing the tissue fragments to settle before aspirating the homogenate.

succinoxidase. These figures are essentially the same as those reported by Hogeboom, Claude, and Hotchkiss [8] for mammalian liver and by Recknagel [16] for ovarian eggs of *Rana pipiens*.

Our failure to recover more than 60 per cent of cytochrome oxidase in the mitochondria of ovarian eggs, in the experiment summarized in Table IV, might be interpreted as indicating an extra-mitochondrial locus for the enzyme. This appears not to be the case, however, for in none of our experiments were we able to detect a significant amount of the enzyme in cell fractions other than mitochondria. To be sure, some activity was occasionally measured in most all of the other fractions, but the total amount was always small in comparison to that in mitochondria. For example, in two experiments on ovarian eggs, the average enzyme activities in yolk, pigment, and microsomes were 2, 5, and 7 per cent, respectively, of the total amount measured. It seems not unreasonable to regard the slight activity in other fractions as reflecting contamination by mitochondria.

Table IV shows that the sum of the activities of the various fractions tested was considerably less than that of unfractionated homogenate. In general, the total recovery of enzyme activity in our experiments was of the order of 75 to 85 per cent. A number of factors may be suggested as responsible for this, such as partial enzyme inactivation during preparation, synergistic action, between two or more components in the embryo homogenate, and loss of enzyme-containing material during fractionation.

That significant enzyme inactivation occurred as a result of fractionation

TABLE IV

Distribution of protein nitrogen and cytochrome oxidase in ovarian eggs.

Fraction	Total N		Cyt. ox.		
	μg	per cent	Q	Total	per cent
Homogenate	35.6	(100)	112	3950	(100)
Yolk	24.4	68	0	0	0
Pigment	1.14	3.2	228	260	6
Mitochondria	2.10	5.9	1120	2350	60
Supernatant	4.75	13.3	58	275	7
Washings	2.65	7.4	—	—	—
Total	35.04	97.8		2885	73

appears very unlikely, because the technique used in our experiments on embryos was identical with that on liver (Tables III and IV). Moreover, at every stage tested, the specific cytochrome oxidase activity of mitochondria was found to be much higher than that of crude embryo homogenate. It was also much greater than for liver mitochondria.

Although one cannot dismiss altogether the possibility of synergism among cell components [17, 13], especially since this was not specifically tested, it seems to us that the chief factor responsible for failure to obtain approximately 100 per cent recovery from the various fractions is the loss of some enzyme-containing material during preparation. In view of the fact that the mitochondrial fraction represents so little nitrogen (either in absolute amount or as percentage of the total nitrogen in the homogenate) and since the mitochondria were washed several times during preparation, it is easy to see how loss from this fraction could occur. In general, the sum of the total nitrogen obtained from the fractions was only 95 to 97 per cent of that in the starting homogenate. If most, or a major portion, of this deficit in protein nitrogen were to represent loss of mitochondria, virtually all of the cytochrome oxidase activity of the unfractionated embryo could be accounted for. We conclude, therefore, that cytochrome oxidase and succinoxidase are localized and concentrated in the mitochondria at all developmental stages. It may be noted in passing that, unlike cytochrome oxidase, the enzyme cathepsin appears not to be concentrated in the mitochondria. Fully 80 per cent of the activity of ovarian egg homogenate is found in the supernatant solution from which yolk, pigment, mitochondria, and microsomes have been removed by centrifugation.

The changes in cytochrome oxidase activity in embryo homogenates and in mitochondria, during the prefeeding period of development, are summarized in Fig. 1. In the embryo (lower curve), the increase in cytochrome oxidase activity appears to follow an exponential course. The present data

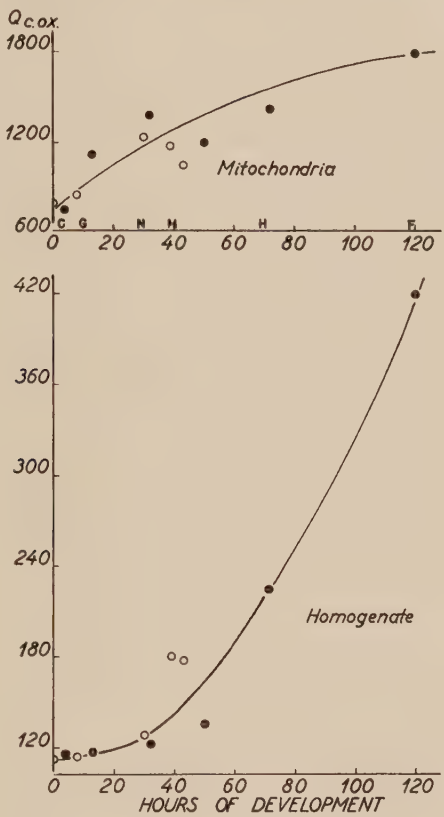

Fig. 1. Graph showing the cytochrome oxidase activity of homogenates and mitochondria of *Xenopus laevis* embryos at various stages of development. The ordinate represents *specific activity of cytochrome oxidase* as mμl oxygen per μg protein nitrogen per hour. The abscissa denotes *developmental stage* in hours from time of fertilization. Values at zero hours were derived from ovarian eggs. Letters on the abscissa indicate embryonic stage as follows: *C*, cleavage; *G*, gastrula; *N*, neurula; *M*, first muscular response; *H*, hatching; *F*, feeding stage. ● and ○ denote eggs from two different females.

thus stand in striking contrast with those of Spiegelman and Steinbach [21] who reported that cytochrome oxidase activity in *Rana pipiens* embryos remains constant throughout development. On the other hand, the development of cytochrome oxidase in *Xenopus* (Fig. 1) is qualitatively similar to the process described for *Amblystoma punctatum* [1, 2]. The chief difference between the two species is quantitative. In *Xenopus*, the enzyme increases approximately four-fold before the onset of feeding; in *Amblystoma*, the increase is of the order of twenty-fold. With both species, however, cytochrome oxidase is at all times greatly in excess of that needed to sustain the respiratory needs of the embryos.

During the developmental span represented in Fig. 1, the amount of new embryonic material constantly increases at the expense of yolk, although the total nitrogen of the embryo remains essentially constant. The change in cytochrome oxidase activity reflects this process of embryonic growth.

If the increase in cytochrome oxidase activity in the embryo were altogether due to multiplication of mitochondria, one would expect to find that the specific activity (Q) of mitochondrial preparations would remain constant during development. The upper curve of Fig. 1 shows that this is far from being the case; the Q value of the mitochondria increases 240 per cent during the period represented by the graph. It is clear that a change occurs in the mitochondrial fraction whose effective result is to increase the cytochrome oxidase activity per unit of mitochondrial protein nitrogen. The nature of this change is at present obscure, but several possibilities suggest themselves. There is a vast difference between the mitochondrion as a cytological entity, on the one hand, and the mitochondrial fraction as a biochemical preparation, on the other. Hence, the change in the Q value of mitochondria shown in Fig. 1 might represent something as simple as the gradual disappearance during development of a contaminant of the mitochondrial fraction. It might also reflect a shift in the "population statistics" of the mitochondrial fraction representing changes in the relative numbers of specific kinds of mitochondria brought about by differential growth of the various tissues of the embryo. That differences in mitochondrial populations might occur during development has been suggested by Lehmann and Wahli [14] for somatoblasts of *Tubifex* embryos. On the other hand, it could represent an intrinsic change in the individual mitochondria characterized by the transformation of catalytically inert protein into cytochrome oxidase. The concept that a biochemically complex and highly organized particle, the mitochondrion, has an "ontogenetic history" is not without attraction to students of development.

SUMMARY

A study has been made of the development of cytochrome oxidase in homogenates and isolated mitochondria of the embryos of *Xenopus laevis*. Cytochrome oxidase activity increases in the embryo throughout the prefeeding period of development. At all stages tested, the enzyme appears to be localized in the mitochondrial fraction of embryo homogenates. During development, the specific activity of the mitochondrial fraction (on a protein nitrogen base) shows a substantial increase.

This work was done at the Carlsberg Laboratory in Copenhagen. We wish to express hearty thanks to Dr. K. Linderstrøm-Lang and Dr. Heinz Holter for extending to us the facilities of the laboratory and for their friendly interest in the progress of this work. One of us (E.J.B.) held a Fulbright Research Award; the other (R.W.) was supported by the Janggen-Pöhn Foundation (St. Gallen, Switzerland).

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STUDIES ON THE YOLK CELL IN *BOMBYX MORI*

IV. GRANULAR STAINING IN THE ALBUMINOUS YOLK GLOBULES

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Granular staining, especially with neutral red, is a rather familiar phenomenon in many animal and plant cells, when they are examined by *in vivo* or *in vitro* staining. Though its real nature has not yet been made clear, and many authors explain their own cases differently [2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 13, 14, 15], it seems that certain intra-cellular, probably colloidal [1, 8, 9, 10, 11], conditions are necessary for the manifestation of granular staining in addition to external conditions, such as concentrations and pH values, etc., of the media in which the cells are examined.

The author has studied this phenomenon in the albuminous yolk globules of silkworm eggs stained with neutral red *in vitro*. The granular staining is manifested in the yolk globules of silkworm eggs as clearly observable in Fig. 1, showing many characteristics similar to those reported by Jägersten [4] in the yolk globules of *Rana*.

In the author's case the manifestation of granular staining is markedly affected by pH values and, when pH is constant, by concentrations of the surrounding media. These relations are seen in Table I.

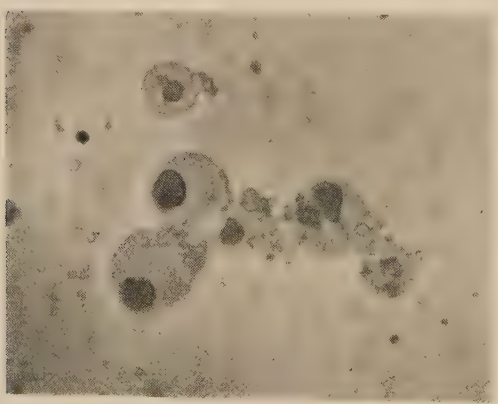

Fig. 1. Granular staining in the yolk globules of the silkworm egg (ca. × 1800).

TABLE I

pH	A J115		B ymn × C110		C J115		D ymn × C110		E J122	
	2.4/15*M*	0.8/15*M*	2.4/15*M*	0.8/15*M*	2.4/15*M*	0.8/15*M*	2.4/15*M*	0.8/15*M*	2.4/15*M*	0.8/15*M*
7.9	+	+	+ +	+	+ +	+	+ + +	+	+ + +	+
7.7	+	+	+	±	+ +	+	+ +	+	+ + +	+
7.1	±	±	+	±	+	±	+	±	+	+
6.7	±	±	±	±	±	±	±	±	±	±

±: very rare
+: not so rare
+ +: below 50 per cent of the yolk globules
+ + +: above 50 per cent of the yolk globules

In this experiment yolk globules were stained with 0.02 per cent (final) neutral red solution mixed with Sörensen's phosphate buffer solutions at the time of use. The molarities given in Table I represent the final concentrations of the buffer solutions. The mixtures cannot stand storage, because in the media of higher pH values precipitation of the dye takes place very quickly. The materials used in this experiment were hibernating eggs of three silkworm varieties which were classified into the five groups (A–E) according to differences in preserving temperature.

The high pH value of media is an important factor as mentioned above, but in the author's case it cannot make appear such granules or droplets in the buffer solutions which contain no dye. As the isoelectric point of the yolk globules is about 6.5 [12], the fact that granular staining is less in the media at pH 7.1 and 6.7 may be explained by their low affinity to the dye.

Effects of the lyotropic series of ions on the granular staining were examined. It was observed that there are upper and probably lower limits to the concentrations of each salt beyond which development of the typical granules is checked or stopped, even though the pH may be favorable. The upper limit is rather clear, but the lower is obscure because many yolk globules are broken in such hypotonic solutions. In the case of cations, K_2SO_4, Na_2SO_4 and $MgSO_4$ were compared as to their upper limits of concentration. Because these solutions had pH values below 7.0 and caused low affinity of the yolk globules to the dye, the results obtained were not clear. But it seems certain that the following order in relation to increasing concentrations of the upper limits exists: $K^{\cdot} < Na^{\cdot} < Mg^{\cdot\cdot}$.

In the case of anions the relation between sodium citrate and sodium

Table II

Final conc. (*M*)	Sodium citrate			Sodium acetate			Sodium tartrate		
	C J115	E J122	D ymn × C110	C J115	E J122	D ymn × C110	C J115	E J122	D ymn × C110
0.40	−	+	+	+	+ +	+	−	−	−
0.32	+	+	+	+	+ +	+ +	−	±	±
0.24	+	+ +	+ +	+	+ +	+	±	±	±
0.16	+ +	+ + +	+ + +	+ +	+	+	±	±	±
0.08[1]	+	+ +	+ +	+ +	+	+	+	+	+

Neutral red was 0.02 per cent.
[1] Many globules were broken in such a hypotonic solution giving less accurate results.

Table III

Concentrations of sodium acetate (*M*)	Days of incubation at 25°C (E J122)				
	1	2	3	4	5
0.40	+ +	±	−	±	−
0.32	+ +	+	+	±	−
0.24	+ +	+ +	+ +	+	±
0.16	+ + +	+ + +	+ + +	+ +	+ +
0.08	+ +	+ + +	+ + +	+ +	+ + +

After the fifth day the yolk globules became easily broken.

acetate could be examined more accurately than in the above-mentioned case of cations owing to higher and nearly similar pH values (7.3–7.7) of both solutions (Table II) citrate < acetate.

Similar results were obtained repeatedly in the other experiments. Sodium tartrate solutions were also tested, but owing to their lower pH values (6.9–7.1) which may cause weak staining (Table I) the results cannot be compared directly with those obtained in sodium citrate and sodium acetate solutions.

Granular staining is also affected by internal conditions of the yolk cell. For example, the staining decreases gradually during the incubation as seen in Table III. In this case hibernating eggs which had been kept at 5°C were incubated at 25°C. It must be noted that changes in temperature which may cause some colloidal changes in the globules to have possible effects on the

staining. But even in the experiments in non-hibernating and artificial non-hibernating eggs which have not undergone such temperature changes, the similar decrease in granular staining during the incubation as in Table III is a common phenomenon.

These results may be expressed in other words, namely, colloid particles in the albuminous yolk globules of silkworm eggs at different stages of development are different in water binding capacity, and when the other conditions are definite they may have different optimum concentrations of media for the manifestation of granular staining. And the data mentioned above show that colloid particles in the yolk globules may decrease in water binding capacity during the incubation of eggs. The differences in the granular staining between the eggs which have different careers (A–E), accordingly are different in dormancy, may also be attributed to the similar differences in intracellular conditions. This relation between the dormancy of eggs and the water binding capacity of colloid particles is an interesting problem of which an account will be given elsewhere.

CONCLUSION

The granular staining in the albuminous yolk globules of silkworm egg is affected not only by the pH value and concentration of the media, but also by intra-cellular conditions among which the water binding capacity of colloid particles seems to be an important factor.

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SERIAL SECTIONS OF VACCINIA VIRUS EXAMINED AT ONE STAGE OF DEVELOPMENT IN THE ELECTRON MICROSCOPE

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In a previous communication [1] intracytoplasmic particles of vaccinia virus were shown at one stage of their development to contain a dense nucleus-like body (nucleoid) separated from granular material (viroplasm) by a zone of lesser density. In order to obtain further information concerning the structure of this viral form, consecutive serial sections have been examined in the electron microscope. Two representative examples of such serial sections are illustrated and discussed in the present communication.

MATERIALS AND METHODS

Chorioallantoic membranes of chicken embryos were infected, harvested, fixed in isotonic, buffered 1 per cent osmium tetroxide, dehydrated in ethyl alcohols and embedded in the manner previously described [1]. Consecutive sections[1] were cut on a microtome designed by Porter and Blum [2]. The ribbons of attached sections were floated onto the surface of a 25 per cent acetone–75 per cent water mixture and picked up directly on formvar-coated grids. The grids, Sjöstrand type, consisted of parallel slits permitting consecutive examination of the serial sections. The observations were made with an RCA type EMU-2E electron microscope.

RESULTS

Fig. 1 shows part of an ectodermal, epithelial cell containing intracytoplasmic viral particles. The nucleus of the host cell occupies the right portion of the figure and the cell border traverses the lower third of the field diagonally. The cytoplasm has undergone early vacuolization and contains besides characteristic virus several distorted and probably disintegrating mitochondria. The field illustrated by serial section VII in Fig. 2 is outlined.

Figs. 2 and 3 illustrate eight consecutive serial sections of vaccinia viral

[1] The authors wish to thank Miss Dorothy Gelber for cutting the sections. The technical assistance of Miss Ermalee Grant and Miss Florence Wu is also gratefully acknowledged.

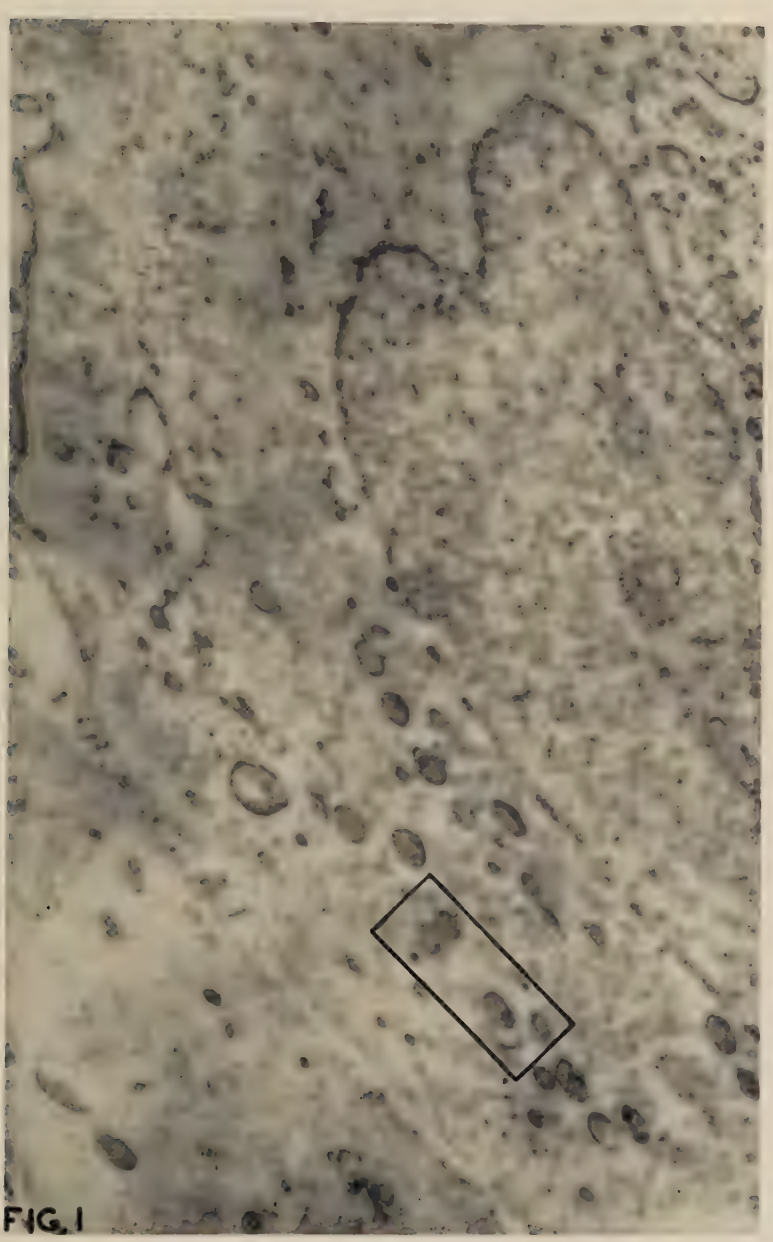

Fig. 1. An epithelial cell in the ectoderm of the chorioallantoic membrane. The nucleus occupies the upper right portion of the field while the cell membrane traverses the lower left diagonally. The area included in Fig. 2 at the level of section VII is outlined.

particles in the cytoplasm of two different cells. Particles *A* and *E*, Fig. 2, are sectioned through their entire thickness appearing in 7, and 6 or 7 sections, respectively. Particle *H*, Fig. 3, is sectioned nearly all the way through, appearing in 8 sections. The remaining particles lie in such a plane that these serial sections do not permit examination at all levels, although it should be noted that particles *D*, Fig. 2 and *I*, Fig. 3, are each transected six times. The maximum major and minor axes of particles *A*, *E* and *H* measure 280 × 160 mμ, 290 × 150 mμ and 300 × 150 mμ, respectively. It is probable that the viral particles were spheres before sectioning, their present shape reflecting compression by impact of the knife. The diameters of spheres compressed into the forms measured above can be calculated to be 232 mμ for particles *A* and *E*, and 238 mμ for particle *H*. Assuming particles *A* and *E* were sectioned seven times and particle *H* eight times, the average thickness of the sections in Fig. 2 is 33 mμ and in Fig. 3 is 30 mμ. It should be emphasized, however, that the opacity of the methacrylate to the electron beam and the relative density of the structures observed indicate considerable variation in thickness of individual sections. Thus, in section I of Fig. 2 the density of the viroplasm and viral membranes approximates that of cytoplasmic fine components and even approaches the diffuse opacity produced by the formvar and methacrylate. Section I, therefore, can be said to be much thinner than section II; and, as is often the case with very thin sections, it exhibits more compression. In the same figure section VI is somewhat thinner than section IV or V, while in Fig. 3 sections I, IV and VIII are thinner than those adjacent to them.

The apparent width and relative density of the limiting membrane of each viral particle depends in part on the angle at which it lies within the section. Sections transecting the membrane obliquely near the periphery of the virus cause it to appear wide, indistinct and less dense, as shown by particle *A*, section III, and particle *E*, section IV, in Fig. 2; or by particle *H*, section VI in Fig. 3. The thickness of the section, however, modifies its appearance as well. Thus section VI in Fig. 2 transects particle *A* nearer its periphery than section V, yet the limiting membranes appear to be of similar width. Because section VI is thinner, the membrane, although lying more obliquely, traverses no greater distance before passing out of the plane of section. Similar effects of section thickness on the apparent

Fig. 2. Consecutive serial sections of intracytoplasmic virus. Particle *A* has been transected 7 times, particle *D*, 6 times. Whether the eccentrically sectioned particle in the lower third of section II represents particle *C* or *E* is impossible to determine. Magnification 60,300 ×.

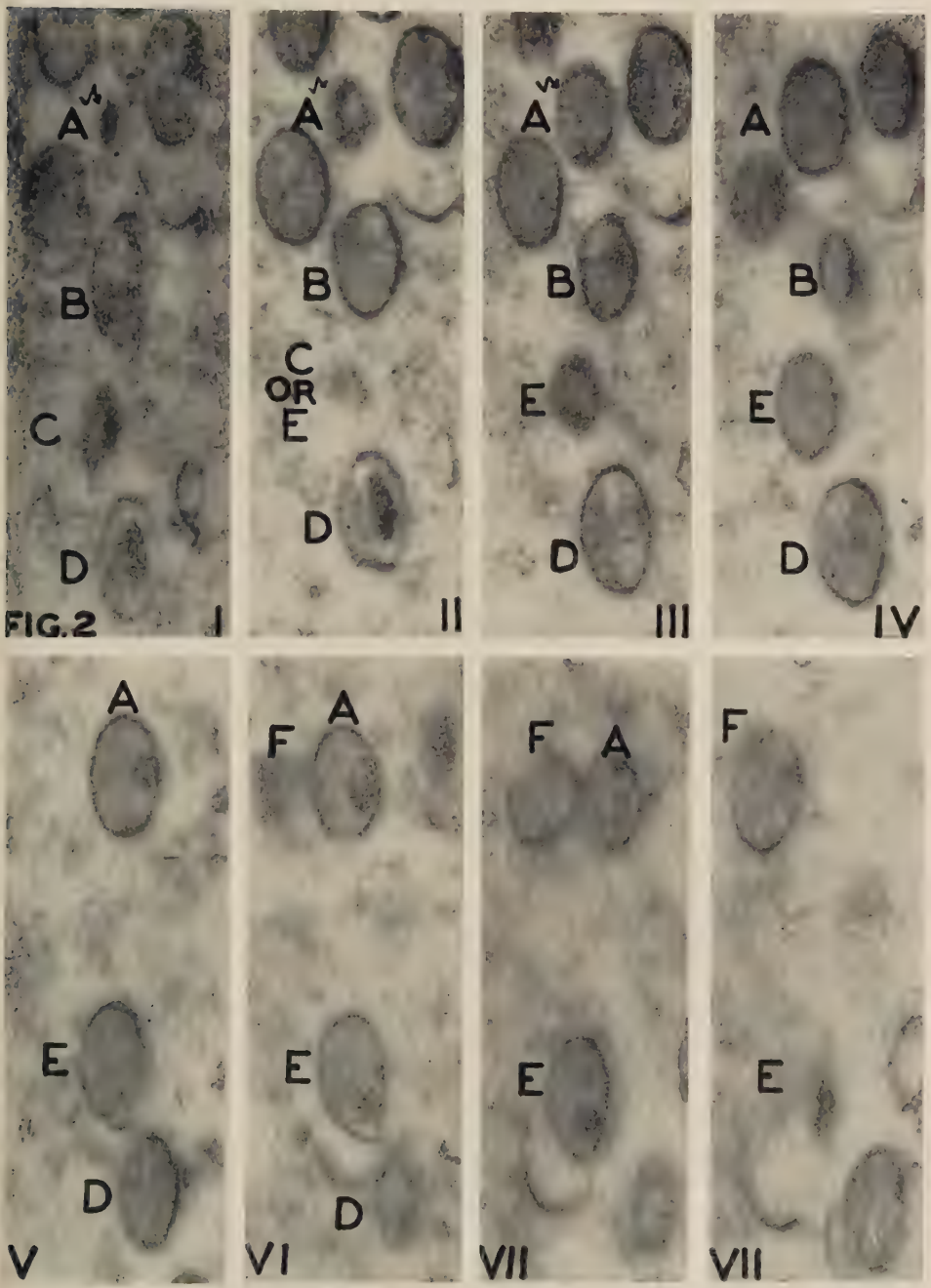

FIG. 2

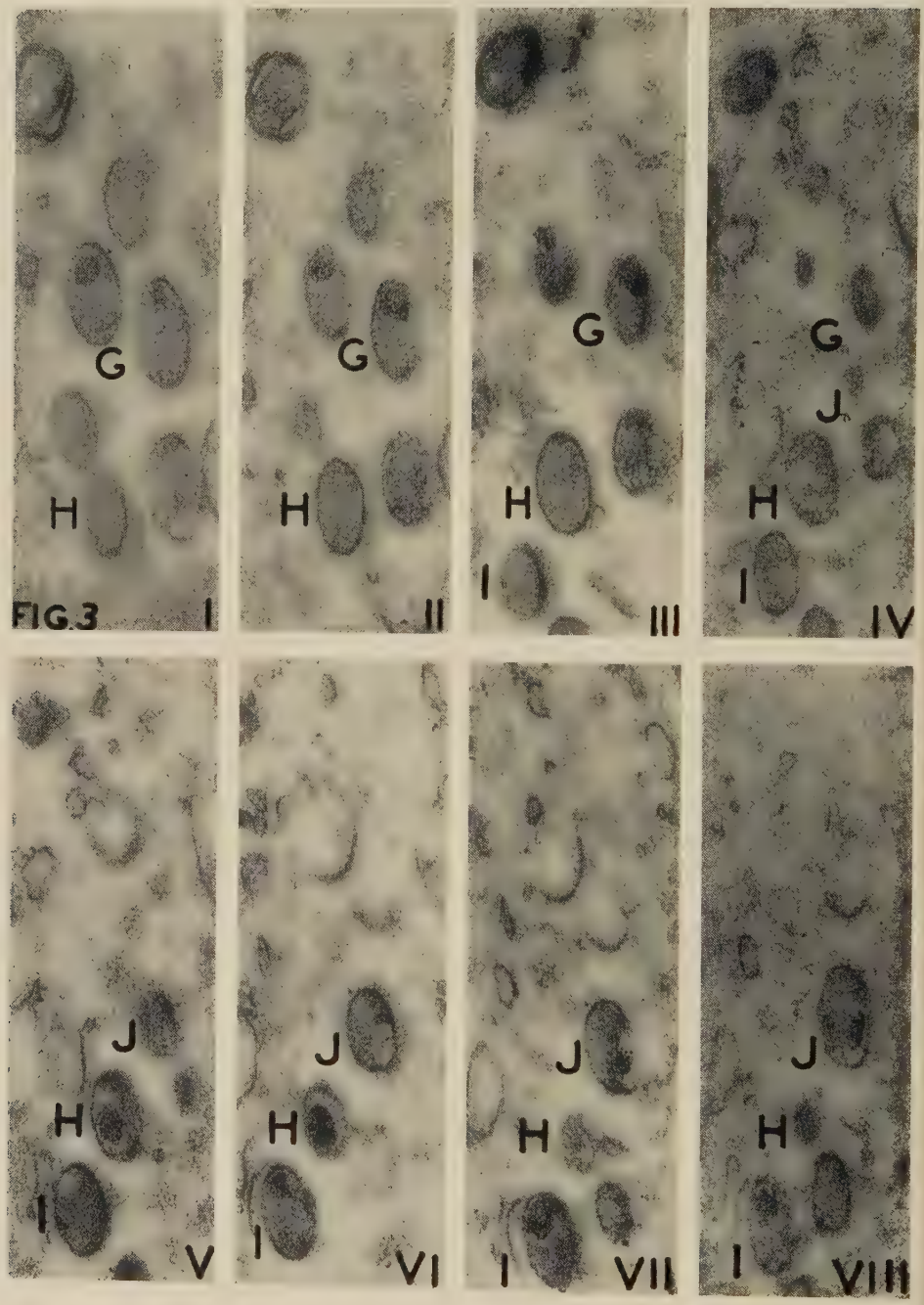

FIG. 3

width of membranes can be observed in particle *B* by comparing the more eccentric section III to the thicker section II, Fig. 2; in particle *H* by comparing the more eccentric section II to the thicker section III, Fig. 3; and in particle I by comparing the more eccentric section IV to the thicker section V, also Fig. 3. The thickness of limiting membranes assumed to be most nearly perpendicular to the plane of section measured 9–12 mμ.

Scattered among viral particles may be seen membranes of generally similar width, but of variable length and shape. No viroplasm is apparent adjacent to the membranes illustrated. Whether they represent fragments of viral membranes or whether they are cytoplasmic components is not yet clear. Within recognizable viral particles localized defects were occasionally encountered, representative examples of which may be seen in particle *E*, section V, and particle *F*, section VII, Fig. 2; and in particle *J*, section VII, Fig. 3. In each instance loss of adjacent fine cytoplasmic components, as well as variable amounts of viroplasm, is evident. That most of such defects did not exist in the tissue before sectioning is indicated by the fact that if they did, a defect of the size encountered in particle *J*, section VII, Fig. 3 should appear in several consecutive serial sections.

The viroplasm is composed of finely granular, moderately dense material and appears to be in contact with the limiting membrane. The dense, granular nucleoid is usually located eccentrically and is ordinarily oval, although elongated forms are occasionally noted, as illustrated by particle *D*, section II, Fig. 2. The maximal dimensions of the nucleoid in particle *E* are 75 × 120 mμ; those in particles *G*, *H* and *I* are 75 × 110 mμ. Although the nucleoid does not appear to possess a membrane it is usually separated from the viroplasm and adjacent limiting membrane of the viral particle by a zone of less density. Occasionally a section transects this zone including little or no nucleoidal material. Particle *D*, section III, in Fig. 2, and particles *H*, *I* and *J*, sections IV, V and VI respectively in Fig. 3 represent such levels of transection.

DISCUSSION

Section thickness and distortion caused by impact of the knife account in part for the difference in definition of membranes and the shape of the viral particles. Close examination of numerous groups of serial sections,

Fig. 3. Vaccinia virus within the cytoplasm of another epithelial cell. Particle *H* appears in 8 sections. Particle *J* in section VII illustrates a characteristic localized defect. Magnification 47,000 ×.

however, reveals other changes in structure as well as variable localized defects which are more difficult to explain. Detailed studies of the effect of the electron beam on thin sections indicate that sublimation and flow of the methacrylate decrease the thickness of the section, modify its contours and alter to some extent the appearance of contained structures. These observations will be published in a separate communication. Nevertheless consecutive serial sections do permit reconstruction of structure in three dimensions.

SUMMARY

Two clusters of intracytoplasmic viral particles are illustrated in eight consecutive serial sections. The sections average 30–33 mμ in thickness. The viral particles are spherical with diameters varying from 200–240 mμ. They possess a dense, limiting membrane 9–12 mμ thick. All particles sectioned five or more times can be shown to contain a nucleoid at one stage of development.

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BRIEF NOTES

A CRITICAL STUDY ON THE INDUCING CAPACITY OF THE DEVITALIZED ORGANIZER

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It has been widely assumed that the induction of the axial system by the organizer in early amphibian development can be ascribed to the action of some chemical substance(s) present in the organizer. This idea emerged mainly from the early observations, made by several workers during 1932–1933, that the organizer is able to induce neural tissue even after it is killed by drying, heating, freezing or treating with ethanol [1, 2, 4]. It appeared logical at that time to infer from this that the inducing capacity of the normal organizer is not connected directly with the living activity but carried by some chemical substance(s) in the organizer. During this study, which was undertaken in an attempt to examine the nature of the induction by the organizer devitalized by mild treatments in order to minimize protein denaturation, some results were obtained which tend to cast a doubt on the above interpretation concerning the inducing ability of the killed organizer.

The experiments were conducted using the embryos of *Triturus pyrrhogaster*, during the breeding season of 1954 and 1955. Either of the following two regions isolated from the mid-neurula (stage 18 of Okada and Ichikawa's table [3]) was employed as an implant after various treatments: (a) neural plate plus underlying archenteric roof except its anterior one fifth and posterior one third (abbreviated NR); (b) posterior one fifth of the neural plate (mesodermal portion, abbreviated PN). The implant following various types of pretreatment was enclosed in two sheets of presumptive ectoderm isolated from the early gastrula (stage 11 or 12a) to test their inducing capacity. Operation and cultivation were done in Holtfreter's solution under sterile conditions. Holtfreter's solution was buffered by $NaHCO_3$ either at pH 7.0–7.2 or at 8.0–8.2 without noticeable difference in the outcome. The explants operated were cultured at 18°C for 8 to 13 days prior to fixation with Bouin's fluid.

Experimental group A, Series 1 and 2.—NR and PN were immersed in 70 per cent ethanol at −18°C for 14–24 hours and 14–27.5 hours, respectively; rinsed with Holtfreter's solution and then grafted into the isolated ectoderm. It was found that neural structures were very rarely induced by the implant thus prepared, i.e., in 12 per cent and 5 per cent of the cases, respectively (see Table I). Induction found in these series as well as in other series of the present study was always of archencephalic type.

Experimental group B, Series 3.—NR was immersed in 70 per cent ethanol at 5°C for 19–20 hours, washed carefully with Holtfreter's solution and then used as an

implant. Induction took place in many cases (72 per cent). Comparing this with the results of group A, it is clear that the inducing capacity is markedly influenced by the temperature of the ethanol used for the treatment of the implant. Theoretically two possible explanations for this fact may be offered: (a) the organizer retains its inducing capacity responsible for the normal induction after devitalization, and the subzero ethanol treatment inactivates or decreases this inducing capacity of the devitalized organizer; (b) the devitalization as such deprives the organizer of its inducing capacity, but the ethanol treatment at 5°C produces some change which results in *de novo* appearance of an inducing capacity, while the ethanol treatment at −18°C does not cause such change. The latter alternative may be inconsistent with the ordinary view concerning the induction of the killed organizer. Further series of this experimental group were designed in order to decide between these alternatives.

Series 4.—NR previously treated with ethanol at − 18°C for 20–22 hours was immersed in ethanol at 5°C for 21–26 hours and used as an implant after washing in Holtfreter's solution. As will be seen in Table I, induction took place as frequently as in series 3. This result excludes the possibility that the inducing capacity is irreversibly inactivated or removed by the subzero ethanol treatment. Inactivation produced by the subzero ethanol, if any, must be of a reversible nature. Further if we assume that the effect of the subzero ethanol treatment be a reversible inactivation, then the reversal of this effect must be possible in ethanol at 5°C but not in Holtfreter's solution at room temperature, since in group A, just like in other groups, the implant treated with subzero ethanol was washed in Holtfreter's solution before use.

However, the possibility must also be considered that removal of the inducing capacity occurs after the transfer to Holtfreter's solution but not in the subzero ethanol. This possibility was tested by the next experiment.

Series 5.—NR was treated in the following way: 70 per cent ethanol at − 18°C (21.5–24 hours) → Holtfreter's solution at room temperature (1–2 hours) → 70 per cent ethanol at 5°C (21–21.5 hours). The result showed that frequent induction (73 per cent) is caused by the implant thus treated, contrary to what is expected from the latter possibility.

Series 6.—NR was treated with 70 per cent ethanol first at 5°C for 19–24 hours, then at −18°C for 17–22.5 hours and used as an implant following washing in Holtfreter's solution. As in other series of this group, a high induction frequency (66 per cent) was obtained. It follows that if the action of the subzero ethanol be an inactivation of the inducing capacity of the organizer, this inactivation fails to take place once the organizer has been killed by ethanol treatment at 5°C.

Thus, so far as we keep to the first alternative, that the subzero ethanol inactivates the inducing capacity originally possessed by devitalized organizer, further, complicated assumptions as follows are needed: (a) the inactivation is of a reversible nature; (b) the reversal of the inactivation is caused by ethanol treatment at 5°C but not by the immersion in Holtfreter's solution at room temperature; (c) the subzero ethanol does not exert an inactivating effect on the organizer pretreated with ethanol at 5°C. The first alternative thus appears to be less probable.

On the contrary, the results can be explained far more easily, if we assume the second alternatives, i.e., that the inducing activity responsible for the induction of the living organizer is lost by its death, and that ethanol treatment at 5°C not only devitalizes the organizer but also causes some change which results in a *de novo*

TABLE I

Exp. group	Exp. series	Treatment of implant	No. of cases examined	No. of cases with any induction	Induction frequency per cent	No. of explants including the induction types:							
						Brain	Retina	Nose	Lens	Neural vesicle	Neural mass	Mesenchyme	Pigment vesicle
A	1	NR → ethanol (−18°)	81	10	12	5	2			3		2	
	2	PN → ethanol (−18°)	19	1	5					1			
B	3	NR → ethanol (5°) . .	25	18	72	8	7		2	3		3	
	4	NR → ethanol (−18°) → ethanol (5°) . . .	31	20	65	5	9	1		2	2	3	1
	5	NR → ethanol (−18°) H. sol. → ethanol (5°)	30	22	73	7	7		4	6	1	1	
	6	NR → ethanol (5°) → ethanol (−18°) . .	35	23	66	7	6		2	5	2	3	2
C	7	NR → frozen and dried	34	3	9	3	1						
	8	NR→ frozen and thawed	34	0	0								
D	9	NR → frozen and dried → ethanol (5°) . . .	16	12	75	7	3		1	4	1		
	10	NR→ frozen and thawed → ethanol (5°) . . .	16	8	50	1	4			2	1		

appearance of an inducing capacity, while at −18°C ethanol does not produce such change. If NR has been subjected to the ethanol treatment at 5°C at any step (in group *B*), and such change has once taken place, it shows a strong inducing capacity regardless of whether it is treated with subzero ethanol and at what step the subzero ethanol treatment is done. Since the denaturation of proteins may be considerable in ethanol at 5°C but not at −18°C, it seems probable that the denaturation of protein in the organizer by the ethanol treatment at 5°C is accompanied by the appearance of the inducing capacity. The fact that the induction is rarely found in group A, where only the subzero ethanol was used for the treatment, may be ascribed to the situation that denaturation might not be abolished completely by the subzero ethanol treatment, and/or that some denaturation-like change occurs subsequent to implantation. It may thus be suggested that the organizer loses its original inducing capacity when it is devitalized without accompanying denaturation, but if protein denaturation is caused by the killing procedure an inducing capacity appears.

Experimental group C.—If this assumption be valid, the organizer killed by other means which do not cause appreciable protein denaturation should also have only a weak or no inducing capacity.

Series 7.—NR was frozen by solid CO_2-ether mixture, dried in high vacuum from the frozen state, and used immediately or after storage for one day at −18°C. The

inducing capacity of the frozen dried NR was found to be very weak (induction frequency: 9 per cent).

Series 8. —NR was frozen by soljd CO_2-ethanol mixture, and after 3-5 minutes thawed in Holtfreter's solution at room temperature. So far as examined no induction was obtained by NR prepared in this manner.

Experimental group D.—In this group the inducing capacity of the frozen-dried and the frozen-thawed NR which were afterwards treated with ethanol at 5°C was tested. From the above hypothesis, these materials would be expected to have a strong inducing capacity after ethanol treatment at 5°C.

Series 9. -The frozen-dried NR was immersed first in Holtfreter's solution for about 1 hour, then in 70 per cent ethanol at 5°C for 21 hours and used as the graft after washing in Holtfreter's solution. The implant so prepared was shown to have a strong inducing capacity (induction frequency: 75 per cent).

Series 10. —The NR previously frozen and thawed in Holtfreter's solution was immersed in ethanol at 5°C for 20 hours, washed in Holtfreter's solution and then employed as a graft. As will be seen from Table I, ethanol treatment at 5°C causes the appearance of considerable inducing capacity in the frozen-thawed NR.

All these results are as expected on the basis of the above assumption, thus providing further support for it. It appears therefore highly possible that devitalization as such deprives the organizer of its original inducing capacity, and that the inducing capacity of so called killed organizer is an artefact resulting from the denaturation of proteins in the organizer. This view is also favoured by the following facts: (a) induction by the killed organizer differs in its quality from that of the living organizer, i.e., the killed organizer is able to induce the archencephalic structures alone, whereas the living organizer can also induce deuterencephalic and spinocaudal structures; (b) induction of the same quality as that of the killed organizer is also caused by dead germ regions, which are not inducers in the living state, as well as a variety of chemical substances, some of which are not normal constituents of the cell.

Thus, a doubt may be cast on the main basis underlying the idea that normal induction is mediated by some chemical substance. We have to consider that even if an inducing substance is responsible for normal induction, its action depends in some way on the intact physiological activity of the living organizer cells. The possibility may also be suggested that normal induction is brought about by the propagation of some change in the state of the protoplasm from the organizer to the adjacent ectoderm rather than by the transfer of a specific substance.

The author wishes to express his sincere gratitude to Prof. Tuneo Yamada for his unfailing interest and encouragement as well as for his valuable criticism and advice during the course of this study. Further thanks are due to Dr. Jack Collier for his kind help in preparing the manuscript.

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EINE VERBESSERTE DURCHSTRÖMUNGSAPPARATUR ZUR GEWEBEZÜCHTUNG AUS GLAS

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Die an früherer Stelle [1] beschriebene Durchströmungsapparatur zur Gewebezüchtung war mit dem Ziel entwickelt worden, Zellen ausserhalb des Organismus unter möglichst konstanten bzw. kontrollierbaren Bedingungen über lange Zeiträume so zu kultivieren, dass sie einer mikroskopischen Beobachtung unter bestmöglichen optischen Gegebenheiten stets zugänglich bleiben. Ebenso wie die im folgenden beschriebene verbesserte Ausführung bestand die Apparatur aus einem Vorratsgefäss für das Nährmedium (Abb. 1, *V*), der Untersuchungskammer, in der sich die Zellen befinden (*U*), den beiden Rohrleitungen zwischen Vorratsgefäss und Kammer (*L*), sowie einer Gasleitung mit Wattefilter (*G*). Die Gasleitung mündet kurz unterhalb des Vorratsgefässes in den senkrechten Teil der Mediumrückleitung ein. Die aus ihr aufsteigenden Gasblasen bewirken das langsame Zirkulieren des Nährmediums aus dem Vorratsgefäss durch die Kammer und wieder zurück. Wegen seiner günstigen mechanischen Eigenschaften war bei der ersten Ausführung der Apparatur nichtrostender Stahl verwendet worden, aus dem sich die Beobachtungskammer ohne Schwierigkeit sehr flach von nur 3 mm Tiefe anfertigen liess.

Es hat sich inzwischen gezeigt, dass die Apparatur ohne Aufgabe ihres vorteilhaften prinzipiellen Aufbaues auch vollkommen aus Glas hergestellt werden kann, was in vieler Hinsicht von grossem Vorteil ist. Nur die Beobachtungskammer musste dazu etwas geändert werden und besonders ihre Herstellung bedarf deshalb einer ergänzenden Beschreibung, während die übrigen Teile leicht anzufertigen sind und ihre Anordnung aus Abb. 1 hinreichend klar ersichtlich ist.

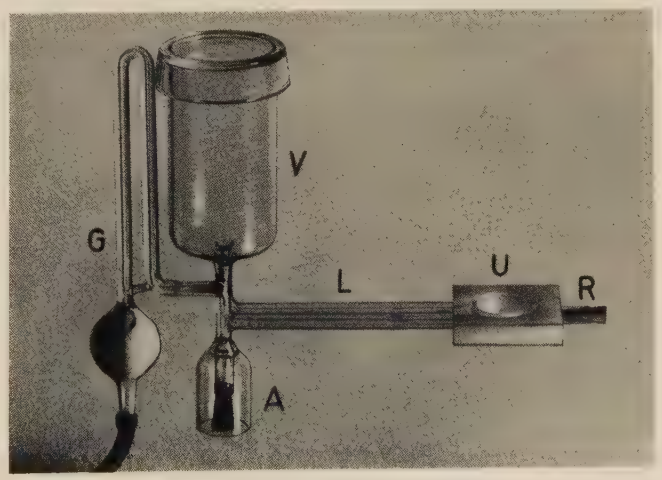

Abb. 1. Durchströmungsapparatur fertig zur Inbetriebnahme ohne Stativ. *V:* Vorratsgefäss, *U:* Untersuchungskammer, *L:* Rohrleitungen zwischen Vorratsgefäss und Kammer, *G:* Gasleitung mit Wattefilter, *A:* Auslass für altes Medium bei Mediumwechsel, *R:* Kurzer Rohransatz an der Kammer, der zum Einbringen des Zellmaterials dient.

Als Ausgang zur Herstellung der Kammer dient ein Glasrohr von etwa 30 mm Durchmesser, in das zwei Glasröhrchen von 1,5 mm Innendurchmesser als zu- und abführende Leitungen zwischen Vorratsgefäss und Kammer nebeneinander eingeschmolzen werden. In gleicher Höhe mit diesen Rohren, jedoch auf der gegenüberliegenden Seite, wird ferner ein 50 mm langer Rohransatz von etwa 4 mm Innen-

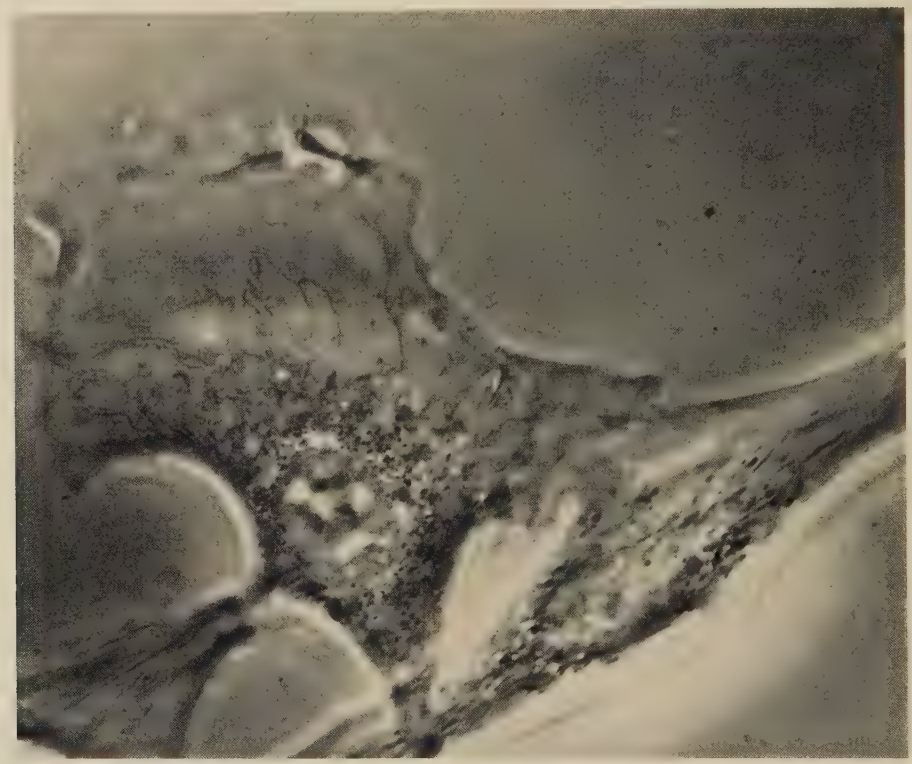

Abb. 2. Lebende Epithelkultur auf Glas (Zungenepithel vom Rind, Stamm SK 2) in einer 6 mm tiefen Kammer. Die Aufnahme wurde mit einem Phasenkontrastobjektiv von 70facher Eigenvergrösserung (Apertur 1,15) gemacht.

durchmesser angebracht. Anschliessend wird das grosse Rohr von 30 mm Durchmesser von oben und unten soweit abgeschliffen, dass nur ein 6 mm hoher, die Kammer bildender Ring mit den drei seitlichen Ansätzen übrigbleibt.

Schwierigkeiten bereitet nun das zum Verschluss der Kammer nötige Aufkleben der oberen und unteren Fensterplatten. Für das die Kammer bildende Rohr lässt sich nämlich normalerweise kein Material von wesentlich mehr als 2 mm Wandstärke verwenden. Nach dem Abschleifen bietet das Rohr den Fensterplatten deshalb eine ringförmige Auflagefläche von nur 2 mm Breite. Dies ist entschieden zu wenig, um die Platten mit Ceresin ohne eine weitere mechanische Spannvorrichtung zuverlässig zu befestigen. Trotzdem kann auf eine solche verzichtet werden, wenn

die Fensterplatten etwas grösser als die eigentliche Kammer sind und der Raum zwischen den überstehenden Plattenteilen durch zwei Stücke eines sterilisierbaren Kunststoffes von der Höhe der Kammer ausgefüllt wird. Durch Einkleben dieser Kunststoffteile wird eine Vergrösserung der klebenden Oberfläche erzielt, die erfahrungsgemäss ausreicht, um die Kammer zuverlässig zu verschliessen.

Während die Tiefe der Kammer ursprünglich bei der ersten Ausführung in Stahl nur 3 mm betrug, musste sie bei der Herstellung aus Glas auf 6 mm erhöht werden. Demzufolge beträgt der Abstand beim Mikroskopieren zwischen Objektiv und Kondensor wenigstens 6,5 mm. Trotz dieses relativ grossen Abstandes ist es möglich, das lebende Zellmaterial mit Hilfe des Phasenkontrastverfahrens auch mit einem stärkeren Objektiv zu mikroskopieren, wie Abb. 2 belegen soll.

Das Einbringen des Untersuchungsmaterials in die Kammer wurde erleichtert und erfolgt, wie auch bei der vereinfachten Kulturkammer [2], durch den seitlichen kurzen Rohransatz, der nach dem Füllen mit einem Stopfen verschlossen wird.

Die Feinregulierung des die Flüssigkeitsbewegung bewirkenden Gasstromes geschieht nicht wie bei der ersten Ausführung durch ein Nadelventil, sondern mit einem Quetschhahn. Auch ein sehr langsamer Flüssigkeitsstrom lässt sich auf diese einfache Weise durchaus exakt einstellen.

Aus dem mit einer kleinen Glocke abgeschirmten geraden Rohransatz unter dem Vorratsgefäss kann die Nähr- oder Untersuchungsflüssigkeit steril beim Wechseln abgelassen werden. Dieses Rohr dient ebenfalls als Flüssigkeitsauslass, wenn die Apparatur im Durchfluss benutzt wird, d.h., wenn ein bestimmtes Medium die Untersuchungskammer nur einmal durchfliessen soll.

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A VERSATILE TYPE OF PERFUSION CHAMBER FOR LONG-TERM MAINTENANCE AND DIRECT MICROSCOPIC OBSERVATION OF TISSUES IN CULTURE[1]

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Tissue culturists have long been aware of the need for a chamber allowing the cultivation of tissues for extended periods of time which would concurrently (1) permit continuous or periodic replacement of fluid media or withdrawal of samples of media for chemical or other assay and (2) permit direct and continuous microscopic

[1] Supported by Grants-in-Aid from the Helen Hay Whitney Foundation.

observation at all magnifications without at any time or in any way disturbing the initial set-up [1, 7]. The perfusion chambers of others [2, 3, 4, 6] were steps in this direction. Over two years ago we developed a type of perfusion chamber which seems to satisfy the above requirements in every sense. For this reason and also because of the interest displayed by tissue culturists and persons in other related fields to whom it has been described in informal discussions, it seems desirable to publish a brief account of it and its use.

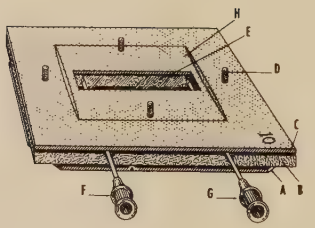

Fig. 1.

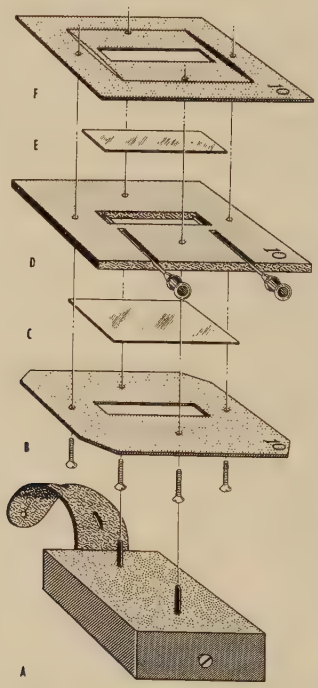

Fig. 2.

Basically, this chamber consists of three matching parts: a $2'' \times 3''$ 18-gauge cadmium-plated metal base plate (Figs. 1*A*, 2*B*), a $2'' \times 3'' \times {}^{1}/_{16}''$ or ${}^{1}/_{8}''$ thick mid-plate of treated [7] pure gum-rubber sheeting (Figs. 1*B*, 2*D*), and a $2'' \times 3''$ 18-gauge cadmium-plated metal top plate (Figs. 1*C*, 2*F*). Each of these three plates has (1) a matching central window or viewing port and (2) four matching screw holes (Fig. 2*B*, *D*, *F*). The screw holes in the top plate are threaded; those in the bottom plate are counter-sunk only. Cadmium-plated flat-headed No. 3-48 ${}^{3}/_{8}''$ machine screws, functioning as compression screws, hold the several plates together (Figs. 1*D*, 2*B*). The window in the rubber mid-plate is closed above and below by glass cover slips or their equivalent (mica or plastic) (Figs. 1, 2*C*, *E*), which are held in place by compression between the top and base plates. The area thus delimited by the window edges of the mid-plate and by the upper and lower cover glasses is the perfusion chamber proper (Fig. 1*E*). Two or more hypodermic needles of suitable size, strategi-

cally located by choice and passing through the edge of the rubber mid-plate into the perfusion chamber proper comprise the affluent and effluent ports (Figs. 1 *F*, *G*, 2 *D*).

In routine practice, the chamber is completely dissembled after each period of use and sterilized by conventional methods. When the chamber is put into use, all of its component parts, except the hypodermic needles, are assembled as indicated in Fig. 2. Item *A* in Fig. 2 is simply a jig which facilitates assembly. The hypodermic needles are best inserted at the time of starting perfusion.

The particular chamber illustrated was designed especially to accommodate the 12 mm × 50 mm coverslips on which tissue explants are grown within conventional roller tubes [6]. The narrowness of these coverslips necessitates (1) a correspondingly narrow viewing port (8 mm × 40 mm) and this (2) necessitates that the edges of the window in the top plate be bevelled and thinned-out to allow the use of high-dry and oil immersion objectives (Figs. 1 *H*, 2 *F*). Except as particular uses demand special sizes and shapes of viewing ports, such as the one illustrated, the viewing port and thus the perfusion chamber proper can be made almost any size or shape. Thus by increasing the area of the viewing port, this type of chamber can be used conveniently with the double or flying-coverslip technique [1, 5, 7].

The thickness of the rubber mid-plate used in this type of chamber is not critical if observations are to be made only with the ordinary light microscope. Its thickness and also that of the upper coverglass are of concern if the chamber is used in combination with phase-contrast microscopy. One-sixteenth inch and $^1/_8''$ thick rubber mid-plates in combination with conventional coverglasses permit the use of the 16 mm, 8 mm and 4 mm long-focus phase-contrast equipment.

The method of circulating fluid nutrient or other perfusate through this type of chamber is one of personal choice. We use, for continuous perfusion, an electrically driven peristaltic action pump (American Instrument Company, Silver Spring, Maryland) with an adjustable flow-rate of 0.2 cc/hr to 8 cc/min.

Perfusion chambers of the type described here have been used in this laboratory in a wide range of studies including drug effects on the growth, contractility, and secretory activity of diverse cells in culture as recorded by time-lapse cinematography and phase-contrast microscopy [8].

We would like to express our thanks to Prof. Ernest Hiser of the Medical Art Department for preparing the illustrations and to Miss Betty Ritcheson, Research Assistant, for especially prompting the preparation of this manuscript.

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THE PRESENCE OF FORMALDEHYDE DURING THE EMBRYONIC DEVELOPMENT OF THE FROG

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The occurrence of formaldehyde in animal organisms has recently been demonstrated [1, 2, 5].

We have used Nash's method [4] to determine the formaldehyde content of frog embryos at various stages of development (*Rhacophorus schlegelii* var. *arborea*).

A homogenate (1 : 9) of fifty eggs at each stage was extracted with 5 per cent trichloracetic acid. The extracts were adjusted to pH 5.5–6 and treated with Nash's reagent B. The extinction of the colour produced had a smooth maximum at 412 mμ. The absorption maximum disappeared by treating the extract with sodium bisulfite or dimedone.

The results obtained which are presented graphically in Fig. 1 show that in the embryonic development of this frog the content of formaldehyde is at a maximum in the gastrula stage.

When the homogenate from eggs was incubated at 37°C for 50 minutes with a solution of glycine, the formaldehyde content decreased as shown in Table I. The

Table I

Influence of glycine and other amino acids on the formaldehyde content of amphibian egg homogenates.

	Rhacophorus schlegelii var. *arborea**	*Rana nigromaclata***		
	Blastula	8 cell	Blastula	Gastrula
Number of eggs in homogenate (1 ml)	10	24	24	17
Amino acids	HCHO μM	HCHO μM		
Glycine	−5.9	−0.8	−2.3	−3.2
Serine	−0.8	+0.5	−0.1	+0.1
Formate	−0.8			
Methionine	−0.4			
Creatine	+2.5			

* 2 ml contained: substrate 2.5 mM, phosphate buffer 50 mM (pH 7.4), homogenate 1 ml, 37°C, 50 minutes.

** 2 ml contained: substrate 5 mM, phosphate buffer 2.8 mM (pH 7.4), homogenate 1 ml, 37°C, 50 minutes.

formaldehyde consumption following addition of glycine was different depending upon the stage of the embryo. Serine, formate and methionine influenced the formaldehyde content of the eggs only very slightly. The increase of formaldehyde upon addition of creatine needs further investigation.

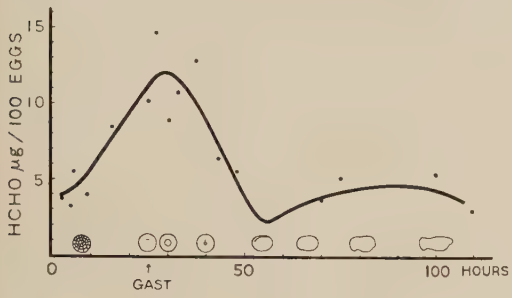

Fig. 1. The presence of formaldehyde during the embryonic development of the frog (*Rhacophorus schlegelii* var. *arborea*) was investigated with homogenate prepared from 50 eggs in each stage.

It was shown by paper chromatography that glycine was the only free amino acid present before the neurula stage of *Rhacophorus schlegelii* var. *arborea* and *Bufo vulgaris japonicus*. For use in paper chromatography the solution deproteinized with trichloracetic acid was neutralized, evaporated and treated with pyridine by the method of Malpress and Morrison [5].

The authors are grateful to Prof. T. Ishikawa for his constant advice and helpful criticism.

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THE ACTION OF DESOXYPYRODOXINE ON CHICK FIBROBLASTS *IN VITRO*

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Desoxypyrodoxine is known to be a potent pyrodoxine analog when given to animals on diets low or deficient in pyrodoxine. Ott first demonstrated this phenom-

[1] This work was supported by the Martha O. Freas Memorial Grant-in-Aid for Cancer Research of the American Cancer Society.

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enon in chicks [4]. He further noted that a ratio of one mole of the vitamin to two moles of the analog was sufficient to prevent the onset of deficiency symptoms. DeRenzo has demonstrated a similar response in the mouse [2] and Emerson in the rat [3]. Both of these animals are considerably less sensitive than the chick.

Very little information is available concerning the effect of desoxypyrodoxine on cells either *in vivo* or *in vitro*. This investigation was undertaken to describe changes in fibroblasts *in vitro* which have been treated with low concentrations of desoxypyrodoxine. Later an attempt was made to reverse the observed changes in the fibroblasts by use of either the vitamin alone or the coenzyme in which the vitamin is known to be present.

Materials and Procedure.—The fibroblasts used in this investigation were obtained from stock cultures grown in a coagulum of chicken plasma and chick embryo extract. The cells were originally explanted from the periosteum of the long bones of 7-day chick embryos. The fibroblasts were explanted from the stock culture onto a round number 0 cover slip. A minimal amount of plasma and extract was used so as to reduce the size of the coagulum. After sufficient time had elapsed for the explant to show a small amount of growth, the cover slip with the culture was transferred to a perfusion chamber [1]. The chamber with the culture was then perfused for 24–48 hours with a control medium consisting of a mixture of 20 per cent horse serum and 80 per cent Tyrode solution. At the end of this period the cells were examined by phase contrast microscopy and photographed. Immediately after this the fibroblasts were perfused with a mixture consisting of varying concentrations of desoxypyrodoxine hydrochloride dissolved in Tyrode solution plus 20 per cent horse serum. The desoxypyrodoxine-Tyrode solution was sterilized by filtration through a Selas filtering candle.

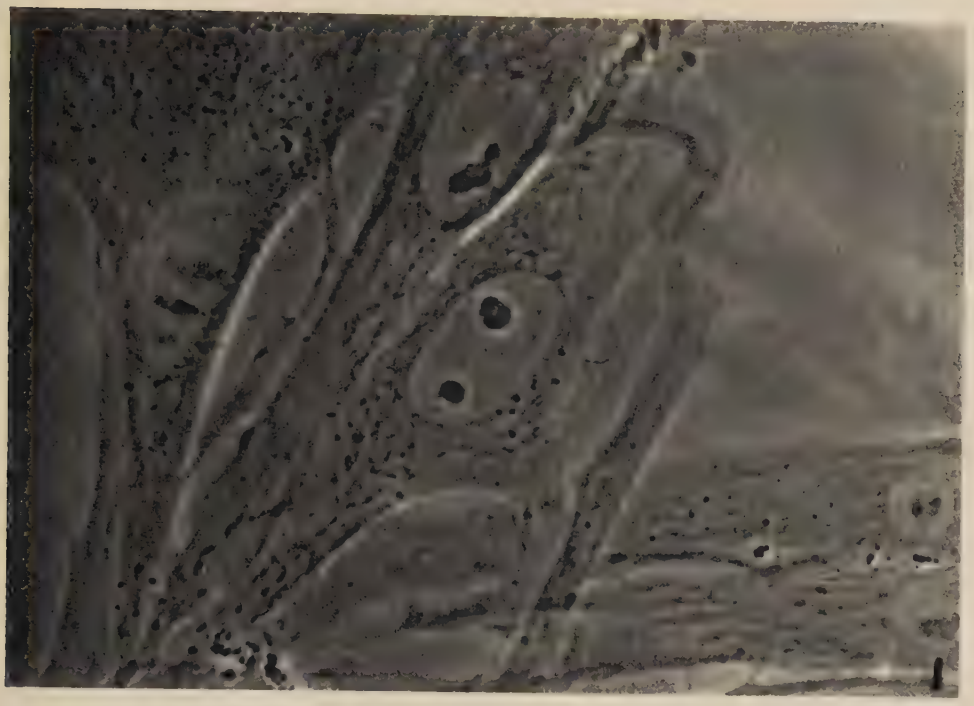
The cells were then photographed at various time intervals to detect any changes which might occur. At the end of each experiment a large number of photographs were taken to assure that the changes observed were consistent throughout the culture.

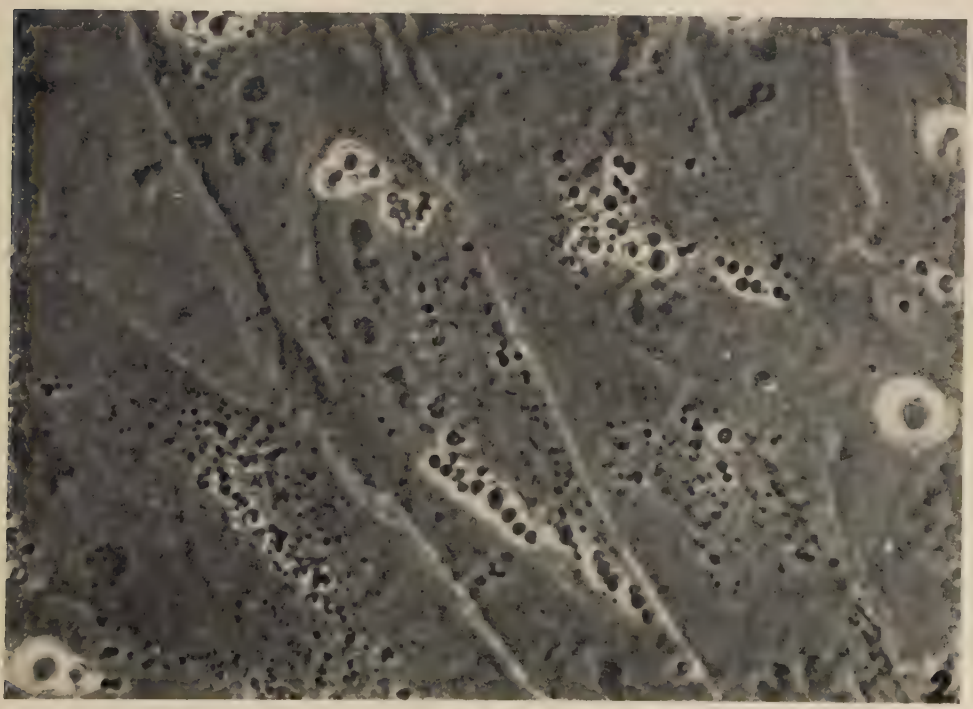
The concentrations of desoxypyrodoxine hydrochloride used were 20, 30, and 40 mg per cent. It was found, however, that a concentration of 20 mg per cent was sufficient to give a maximal cellular response with the least irritation to the fibroblasts.

Results and conclusions.—Desoxypyrodoxine hydrochloride caused no early changes in the fibroblasts. At the end of from 48–96 hours of perfusion however, there was a great increase in the relative size and number of nucleolar bodies within the nucleus (Figs. 1 and 2). In addition there appeared to be an elongation of the nucleus itself with a slight decrease in nuclear size. Some small vacuolar bodies appeared in the cytoplasm.

As perfusion with desoxypyrodoxine continued there was a slowing of the movement of the mitochondrial elements within the cells. This process continued until there was no apparent activity in the cytoplasm. The fibroblasts did not assume the usual appearance of dying cells but rather seemed to be in a "cytostatic condition". Only those cells which had previously produced multiple nucleoli reached this state.

Fig. 1. A normal control fibroblast after 48 hours of perfusion with control medium. ×970.
Fig. 2. A fibroblast from the same preparation after 60 hours of perfusion with 20 mg per cent of desoxypyrodoxine hydrochloride. Note the changes in the nucleoli. ×970.





A return of the cells to the control perfusion medium did not restore the activity of the fibroblasts even after long periods of perfusion.

Additional amounts of the vitamin, when added to the cell medium after treatment with desoxypyrodoxine, also failed to reverse the observed effects.

Umbreit has shown that desoxypyrodoxine is first phosphorylated and then competes with pyrodoxal phosphate for its apoenzymes [5]. Therefore, it was decided that pyrodoxal phosphate should be added to the medium to determine any effects it might have on the static cells. The addition of 20 to 30 mg per cent of pyrodoxal phosphate to the medium seemed to restore only part of the cellular activity. Since pyrodoxine could conceivably be present in other enzymes which do not require that the vitamin be phosphorylated, this result could be expected. Further investigation is being undertaken to elucidate this aspect of the problem.

As the perfusion preparations age, there seems to be a tendency for desoxypyrodoxine to cause a fragmentation of the nucleus. Many small fragments of nucleus appear in the cytoplasm; each usually contains a bit of nucleolar material.

In earlier periods of perfusion with desoxypyrodoxine, mitosis continues at a near normal rate in the cultures. Feulgen stained preparations indicate that this process proceeds in a normal manner. Mitotic activity decreases as the other cellular processes previously mentioned decline in activity.

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HISTOCHEMICAL REACTIONS FOR ELECTRON MICROSCOPY: ACID PHOSPHATASE

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Observations with the electron microscope on sectioned biological material have been confined, for the most part, to descriptions of cellular ultrastructure. At the same time, the precise localization of histochemical reactions has been limited by the

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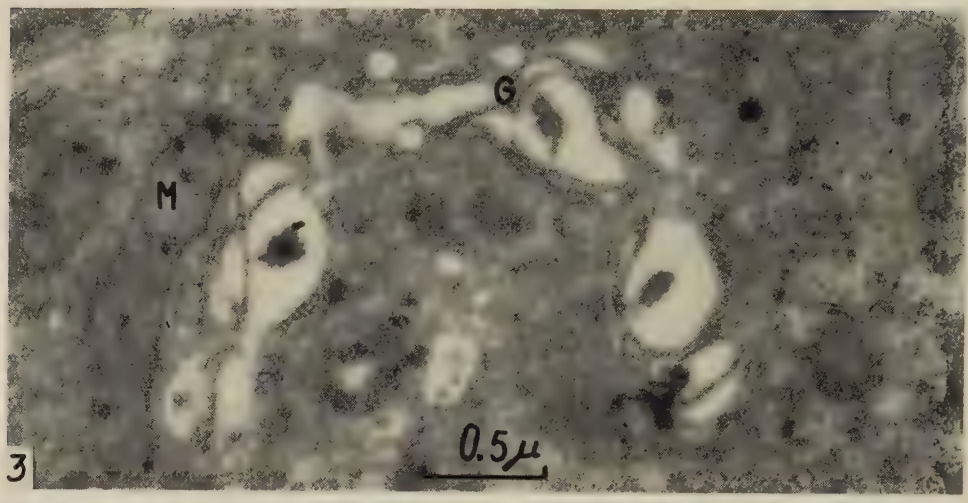
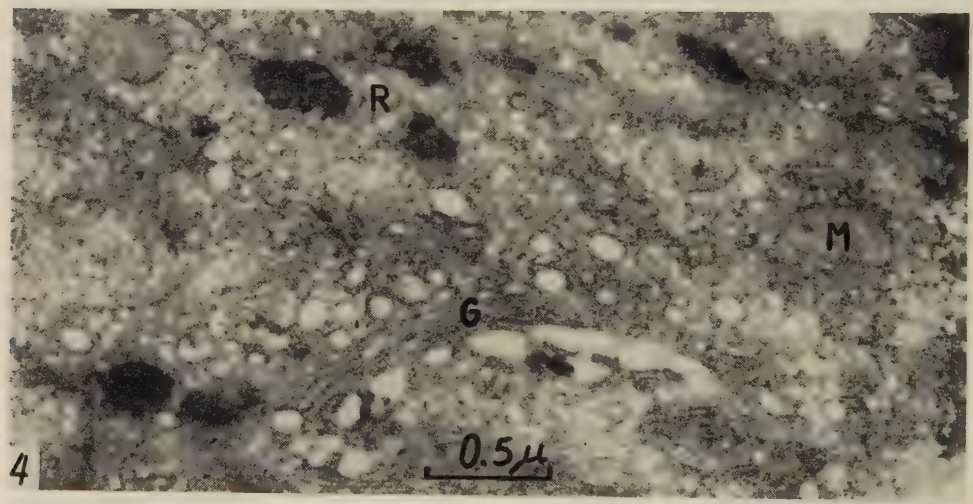
Fig. 1. Mouse intestinal epithelium fixed 2 hours in 1 per cent isotonic osmium tetroxide solution showing brush border (*B*), Golgi complex (*G*) and nucleus (*N*). Magnification 9,000 ×.
Fig. 2. Mouse intestinal epithelium fixed 8 minutes in 1 per cent isotonic osmium tetroxide solution and incubated 16 hours in substrate. Lead sulfide precipitate in brush border and juxtanuclear zones. Magnification 11,000 ×.
Inset: control incubated in the presence of 0.026 per cent sodium fluoride.

B
G
N
1μ
1

N
1μ
2


G
M
0.5μ
3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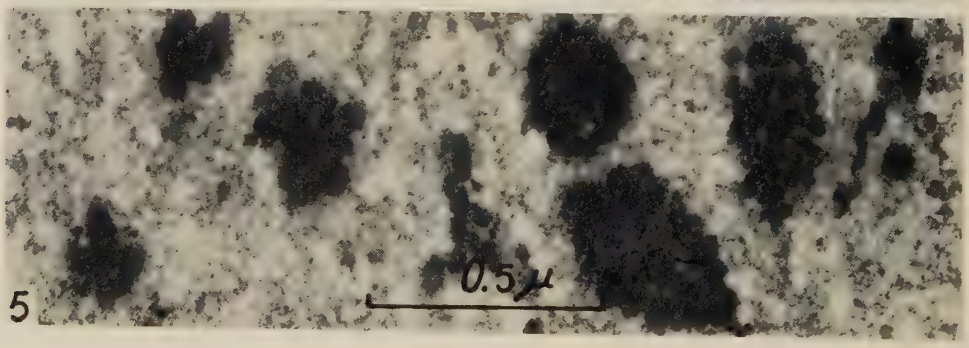
0.5μ
5

resolution of the light microscope. This communication concerns the application of the histochemical technique for acid phosphatase to electron microscopy in an effort to learn more about the structures revealed with both methods.

While the demonstration of acid phosphatase (phosphomonoesterase II) in the intestinal epithelium by the method of Gomori [4] shows the same general distribution as alkaline phosphatase (phosphomonoesterase I), it has been accepted that at least two separate substances are responsible for the reaction rather than a single enzyme capable of phosphatase activity over a wide range of pH. This conclusion is based not only on the difference in the optimal pH range for the enzymes, but on differing sensitivity to enzyme-inhibiting agents (cyanide, fluoride), on different solubilities (alkaline phosphomonoesterase being salt-soluble and acid phosphomonoesterase water-soluble), and on the distribution of histochemical reaction sites in tissues other than the intestine [5]. Light microscope investigations on mouse intestinal epithelium incubated in acid phosphatase substrate demonstrate a strong reaction in the brush border zone of cells and in the supranuclear zone [3].

An electron microscope study at high resolution (better than 30 Å) of the intestinal epithelium of the mouse has demonstrated that the brush border is composed of minute parallel cylindrical projections of cytoplasm about 1 micron long and 0.1 micron wide. These projections are bounded by the cell membrane which appears as two dense lines each 40 Å thick separated by a non-dense area of 25 Å. The cell membrane is an unbroken unit covering each projection and continues to adjacent projections without any discontinuities either at the apices or bases of the projections [8].

The supranuclear zone of these cells includes not only the relatively well-defined Golgi complex [2, 7], mitochondria, and other membrane-bounded bodies but most components of the cytoplasm as well. The Golgi complex of the intestinal epithelial cells lies in close relation to the distal pole of the nucleus and appears as paired dense membranes about 60 Å wide; the membranes of a pair are often separated by large and small vacuoles. Granules of varying sizes and density may lie close to the membranes. The cytoplasm is made up of at least four distinct components which are arranged in various patterns. These basic elements are an homogenous ground substance of varying density, granules about 150 Å in diameter which occur singly or in groups, membranes with attached granules and vacuoles of varying dimensions and densities [8].

For these experiments, in order to avoid destruction of the enzyme by the osmium tetroxide fixative routinely used for electron microscopy, tissues were fixed briefly (eight minutes or less) in 1 per cent buffered isotonic osmium tetroxide, washed in Tyrode's solution, and incubated in Gomori's acid phosphatase substrate at 37°C for periods up to sixteen hours. The tissues were rinsed in Tyrode's solution, washed for two minutes in dilute acetic acid, and then treated with ammonium sulfide. This

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Fig. 3. Juxtanuclear zone of mouse intestinal epithelial cell fixed 2 hours in 1 per cent isotonic osmium tetroxide solution showing mitochondria (*M*), Golgi membranes (*G*), vacuoles and granules. Magnification 32,000 ×.

Fig. 4. Juxtanuclear zone of mouse intestinal epithelial cell treated as Fig. 2, showing mitochondria (*M*), Golgi membranes (*G*), vacuoles, and reaction sites (*R*). Magnification 32,000 ×.

Fig. 5. Reaction sites enlarged to show grapelike clusters of round dense bodies. Magnification 64,000 ×.

treatment results in an end product of lead sulfide which is visible in electron micrographs as a dense homogenous precipitate. The tissues were routinely dehydrated in graded ethyl alcohols and embedded in methacrylate before sectioning with a Sjöstrand microtome [6]. Controls were run by incubating tissues in substrates containing 0.026 per cent sodium fluoride.

Electron micrographs showed the reaction sites as a dense homogenous precipitate distributed in the intercylindrical spaces of the brush border projections (Fig. 2). In sections through cylindrical projections tangential or parallel to the cell surface the precipitate was arranged in a honeycomb pattern, the cylindrical projections appearing non-dense and the interspaces dense. This arrangement is similar to the appearance of the reaction for alkaline phosphatase [1]. In the juxtanuclear zone the precipitate appeared as grape-like clusters of round dense bodies, each body being about 500 Å in diameter. Neither the membranes nor the large vacuoles of the Golgi complex appeared to be involved in the reaction (Fig. 4). Fluoride-inhibited controls showed a normal structure without the lead sulfide precipitate in either the brush border or juxtanuclear zone, eliminating the possibility of a false positive reaction (Fig. 2, inset).

Preliminary observations do not clarify the precise relation of the reaction sites to the cell surface membrane of the brush border zone, nor is it yet apparent exactly which of the many components of the juxtanuclear zone represent the sites of reaction. It is hoped that future work will permit more complete identification of the sites of reaction.

The authors would like to express their gratitude to Dr. F. S. Sjöstrand for the privilege of working in his laboratory.

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THE LABELLING OF MOUSE SPERM BY ADENINE-8-C-14

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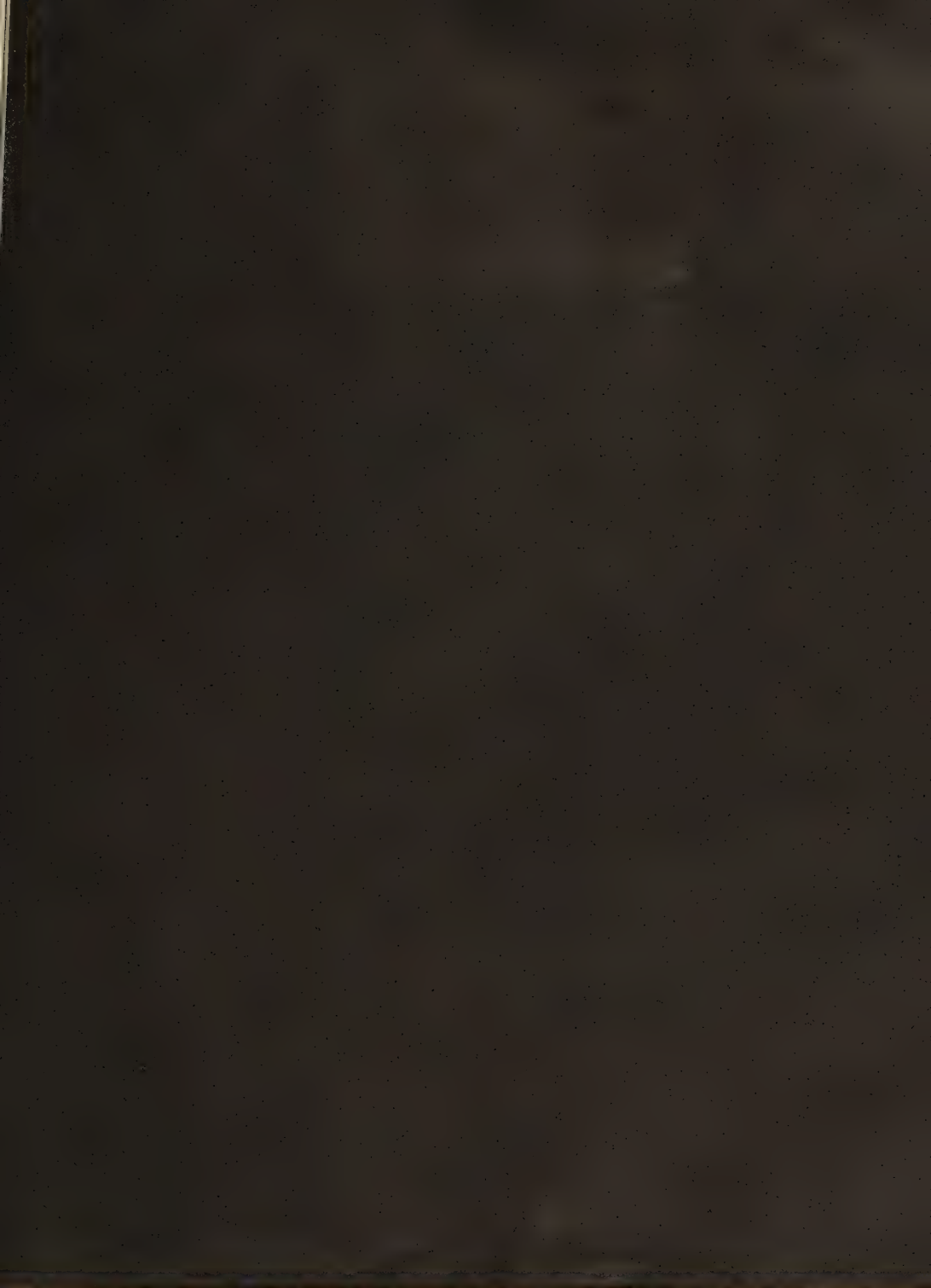
Received October 5, 1955

Data published by several authors suggest the possibility of labelling sperm [2, 3, 5, 7]. The present paper describes a method of labelling sperm, scored by the use of autoradiography.



Figs. 1 and 2, pp. 597 and 598: The two figures were accidentally interchanged during printing. Fig. 1, entitled "Autoradiographs on mouse sperm . . ." should show the sperm-heads with their autoradiographs. Fig. 2, entitled "The percentage of labelled sperm found at various intervals after the injection of adenine-8-C-14 . . ." should show the graph.

Adenine-8-C-14 sulphate (specific activity of adenine: 4.6 mc/m*M*) was injected intraperitoneally into male mice. The activity injected was slightly less than one microcurie per gram of body weight. Injected mice were of three age groups: 3 weeks, 2 males (strain CBA/Be-*a*, average weight 9.2 g); 6 weeks, 2 males (strain C3H, average weight 22.4 g); 6 months, 3 males (strain C3H, average weight 33.1 g). Two control males, aged three weeks and six months respectively, were injected with

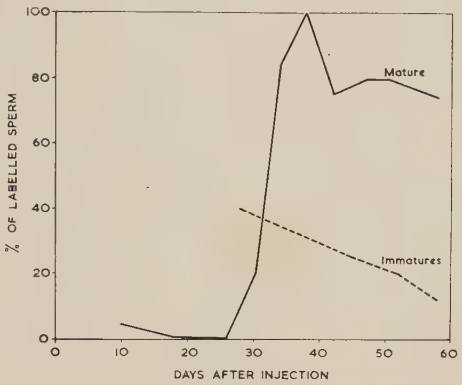


Fig. 1. Autoradiographs on mouse sperm; at least 12 silver grains per sperm can be seen in the overlying emulsion (× approx. 1250).

carrier adenine. Mating of the three week old males commenced at maturity; the other two groups were mated from four days post-injection (p.i.). Superovulated [8] or oestrous females were paired with the males every alternate four days up to 80 days p.i. Mating did not always follow; two of the five mature males seemed unwilling to mate on occasion. Males injected when immature sometimes failed to deposit sperm in the female, but this was also observed in controls.

Females with vaginal plugs were killed approximately 12 hours after mating. Uterine sperm was smeared on gelatinised slides and the fallopian tubes were preserved for study of the ova. Some smears were fixed by heat and alcohol; others by alcohol alone. After rinsing, the smears were coated with Kodak stripping film, exposed, and processed. They were then stained with celestine blue, mounted dry, and examined under a phase contrast microscope for the presence of an autoradiograph (ARG). This procedure ensured clear observation of the sperm and ARGs. For estimates of dosage and incorporation, grains were counted in some samples. Altogether, about 200 ARGs were prepared.

All the males gave labelled sperm (Fig. 1). ARGs were restricted to the sperm heads; none could be associated with the tails. A percentage of labelled sperm occured in the first samples of the youngest males, though ARGs were weak. With successive matings, the percentage and the amount of label per sperm declined rapidly (Fig. 2).

Of the five mature males, three mated regularly. Labelled sperm began to appear from the three males at about 28 days p.i.; the highest percentage occurred at about 38 days p.i., and declined later (Fig. 2). The maximum for each male was 100, 95, and 85 per cent of labelled sperm respectively. The intensity of the ARGs was correlated to the percentage, and was also greatest at 38 days. The two remaining males mated only intermittently; they also gave labelled sperm after 28 days p.i. In the

Fig. 2. The percentage of labelled sperm found at various intervals after the injection of adenine-8-C-14. The graph of the immature males is the mean of two males; only one male is represented in the "mature" graph. The mature male was injected at age 6 months, the immatures at age 3 weeks.

lightly labelled samples the variability of label in the sperm appeared greatest. Deformed sperm were rare.

The uptake of tracer during spermatogenesis was studied. The testes of males injected when three weeks old were fixed at 10 and 17 days p.i., sectioned at 5μ, and ARGs made of them. At 10 days p.i., nuclear and cytoplasmic ARGs were markedly concentrated on spermatogonia, present in spermatocytes, and almost absent on spermatids and maturing sperm. At 17 days p.i., the amount of tracer in the tissue was lower. The tracer was incroporated in all stages up to maturing sperm, though ARGs on the earlier stages were more frequent. From these data the maturation of spermatogonia into mature sperm takes at least 17 days. Howard and Pelc [3] determined that the interval between spermatogonial metaphase and immature sperm was greater than 10 days.

The radiation dose received by the injected males, assuming even distribution and complete retention of tracer, was calculated as 3 reps/day. Determinations made on testes of males injected at age 3 weeks gave actual lower values at 10 and 17 days p.i. According to the data on total incorporation of tracer, the dose received by the mature sperm in the vasa, was below 20 reps/day.[1] Excretion in urine on the 2nd and

[1] The maximum radiation dose received by the sperm has been estimated as follows. A bunch of sperm heads in the vasa were considered as a structure larger than the range of the C^{14} electrons. For the calculation the sperm were assumed closely packed, with no empty space between them. The approximate volume of a sperm head was taken as $20\mu^3$, and of a tail $7.5\mu^3$. With the assumed arrangement the volume of the heads (radioactive volume) and the volume of the tails (non-radioactive volume) were 73 and 27 per cent of the total volume, respectively. 2.3×10^6 C^{14} atoms present inside a sperm head (see text) equals $0.1787 \times 10^{-9}\mu c$. From these data the resulting activity is 6.5 μc per cc of sperm, and the maximum radiation dose is 20 reps/day.

3rd day p.i. was approximately 1 per cent/day of the activity injected, dropping substantially by the 4th day p.i. Activity in faeces was negligible.

Adenine-8-C-14 is a precursor of the nucleic acids adenine and guanine. As very little, if any, RNA is present in the mature sperm, only DNA purines would be labelled. In samples with the highest label, calculation showed the average maximum incorporation per sperm head to be of the order 2.3×10^6 DNA C^{14} atoms (12 grains, 21 days exposure, 2 grains/electron). If we assume an average content of 3.2×10^9 DNA adenine + guanine molecules per sperm, this represents a ratio of labelling of 1 : 1400. As labelling in the tracer is 1 : 14, the effective labelling was 1 : 100.

It is planned to use labelled sperm in several investigations. First, tracer inside a cell may cause genetic aberrations, either through radiation [4, 6], or by transmutation of the decayed atoms [4]. Some of the C3H males are being test-mated for induced mutations. Secondly, if insemination of females with radioactive sperm permits tracing spermatozoa in and around the eggs at fertilisation, relations between eggs and different types of sperm in sperm mixtures can be studied [1]. Thirdly, aspects of pronuclear growth, cleavage, and early development of the egg may be traced by utilising various labels introduced by the sperm. Labelling of the male pronucleus will also permit identification of gynogenetic versus androgenetic haploid eggs.

We wish to thank Dr. S. R. Pelc for suggesting the use of adenine, and Professor C. H. Waddington, F.R.S., for encouragement during our work. This investigation was carried out during the tenure of grants from the British Council and the British Empire Cancer Campaign (J.L.S.), and from the University of Edinburgh and the Agricultural Research Council (R.G.E.).

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